THE EFFECT OF LIGHT ON A RAT MODEL OF DEPRESSION

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Submitted in fulfilment of the requirements for the degree

MSc (Med) Neuroscience

Faculty of Health Science

University of Cape Town

1 December 2014

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PLAGIARISM DECLARATION

I, **Asanda Mtintsilana (MTNASA001)** hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ACKNOWLEDGEMENT

This study was supported by the National Research Foundation (NRF) of South Africa and the FNB Ted and Mabel Everitt Bursary Fund.

I would also like to thank the following people for their support and contribution to my master's thesis.

Emeritus Professor Vivienne A Russell

Prof, thank you so much for giving me the opportunity to be part of your research team. It has been my greatest honour and privilege to have had you as a supervisor, guiding me, every step of the way. I am truly blessed and eternally grateful to have been part of your laboratory, acquire skills and tools to better my work and myself as a scientist. The role you have played in my project, from its inception, has been invaluable to my learning. Your expertise in neuroscience research, in particular in the field of research I conducted, has been indispensable for my Master's project. Thank you so much for your feedback and guidance towards my thesis write up.

Dr Jacqueline J Dimatelis

These few words in this acknowledgement section, do not measure how much I am thankful to have been co-supervised by you. It is with the highest level of gratitude and blessing to have been co-supervised by you. You have been present every single day each part of my project, from conducting experiments, to waking up in the early mornings to help me with dissections and guiding me in writing my thesis. You have taught me so much about conducting research and I have learnt so much from you in the 2 years of my Master's project. Thank you so much for your incomparable guidance throughout my project. The selfless work you have put into guiding my work until it was exceptional has been the very thing that has pushed me to work harder. I can truly and honestly say that I am better researcher than I was before I worked with you.

You have been one of the sincerest, kindest and caring people I have met and because of those attributes; it has been my greatest pleasure to have had the opportunity to work with you. I continue to admire the hard work you put into everything you do and moving forward, I look forward to working just as hard as you do as I enter the next chapter of my life. Thank you for your continuous support and warmth during my Master's degree, despite making many mistakes, repeatedly!!!

Staff in the Neuroscience laboratory

Thank you so much to Mrs N Ismail and Mr Abdul Karim (AK) Samuels for your assistance with animal work during my Master's project. Thank you for your enthusiasm towards my project and your zeal to help whenever I needed assistance with the rats. Thank you for creating a warm environment to work in; it has been a pleasure working with both of you. Thank you to AK, for also availing himself by ensuring that I had all the equipment needed to do my experiments.

I would also like to thank Mr Damon Nazeem and Mr Charles Harris for assisting me with technical problems, especially setting up the gas for the in vitro superfusion experiment and designing some of the equipment used for the in vitro superfusion experiment. Thank you to Mr Damon Nazeem for also supplying me with all the laboratory equipment needed to conduct my experiments. Your assistance throughout the entire period of my Master's is greatly appreciated.

Students in the Neuroscience laboratory

Thank you to Patricia Swart ("my lab partner in crime") for being an incredible person to work with in the laboratory. Thank you for being a caring, loving person and showing me support whenever I doubted myself. Thank you for being my cheer leader and motivating me to extra work hard and not give up. It has been a blessing to know you and share this journey with you. I will miss our lunch breaks, grocery shopping at Pick n Pay and working together in the laboratory.

I would also like to thank Jurgens van Zyl for helping me with dissections. Thank you for providing a fun working environment, especially with your sense of humour.

I would also like to thank Hayley Tomes, Natasha Hardcastle, Dr Jacqueline Wormersly, Dr Toni-lee Sterley, and Inge Vermeulen and Mauritz van den Berg (in no particular order) for their support during my Master's project. Thank you all, for welcoming me in the laboratory and for willing to help whenever I was uncertain about something.

Family and friends

To my grandmother, mom and aunt including my brother and cousins thank you so much for your continuous encouragement and support throughout my Master's degree. Your encouraging words have cheered me and brought me up during the toughest times. To my closest friends; Lerato Majara, Elizabeth Duke, Ayanda Zide, Thembeka Sineke, Lindizwe Dlomo and Sinayo Sirenya (in no particular order) thank you so much for being my main cheer leaders. Thank you all for being a constant reminder of what I can achieve and believing in me.

Again, thank you to each and every single one of you who nurtured, facilitated and encouraged me to give my best.

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ABBREVIATIONS

%	Percentage
°C	Degrees Celsius
µg	Microgram
µl	Microlitres
µm	Micrometre
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	Serotonin
5-HT $_{2A}$R,	Serotonin receptor subtype 2A
5-HT $_{2C}$R	Serotonin receptor subtype 2C
5-HT$_{1A}$R	Serotonin receptor subtype 1A
5-HT$_4$R	Serotonin receptor subtype 4
5HT-egic	Serotonergic
ACTH	Adrenocorticotropic hormone
AVP	Arginine vasopressin
BDNF	Brain-derived neurotropic factor
BSA	Bovine serum albumin
BST	Bed nucleus of the stria terminalis
CBT	Cognitive behavioural therapy
CCL	Chronic constant light
CNS	Central nervous system
CO_2	Carbon dioxide
CORT	Corticosterone
CPP	Conditioned place preference
CR	Circadian rhythm
CRH	corticotropin-releasing hormone
CRF	Corticotropin-releasing factor
CSF	Cerebrospinal fluid
D1	Dopamine group 1
D1 and D5	Dopamine receptor subtypes of Group D1
D2	Dopamine receptor group 2
D2-D5	Dopamine receptor subtypes of Group D2
DA	Dopamine

DOR	Delta-opioid receptor
DOR KO	Delta-opioid receptor knock out
DPM	Disintegrations per minute
DRN	Dorsal raphe nucleus
DTT	Dithiothreitol
ECT	Electroconvulsive therapy
EEG	Electroencephalogram
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent assay
EPM	Elevated plus maze
ERK	Extracellular signal-related kinase
FSL	Flinders Sensitive Line
FST	Forced swim test
g	Gravitational force
GABA	Gamma-aminobutyryic acid
GAM	Goat anti-mouse antibody
GAR	Goat anti-rabbit antibody
GHT	Geniculo hypothalamic tract
GR	Glucocorticoid receptor
HPA axis	Hypothalamus-pituitary-adrenal axis
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IGL	Intergeniculate leaflet
IPT	Interpersonal therapy
IQR	Interquartile range
KDa	Kilo Dalton
KOR	Kappa-opioid receptor
KOR KO	Kappa-opioid receptor knock out
L/D cycle	Light/Dark cycle
M	Molarity (concentration)
MDD	Major depressive disorder
mg	Milligram
mGLuR2/3	Metabotropic glutamate receptor group two
mM	Millimolar
MOR	μ-opioid receptor
MOR KO	μ-opioid receptor knock out

mPFC	Medial prefrontal cortex
MR	Mineralocorticoid
MRN	Medial raphe nuclei
mRNA	Messenger RNA (ribonucleic acid)
MS	Maternal separation
MS CCL	Maternally separated + CCL rats
MT1	Melatonin receptors one
MT2	Melatonin receptors two
NAc	Nucleus accumbens
NaOH	Sodium hydroxide
NE	Norepinephrine
ng/mg	Nanograms per milligrams
NMS	Non maternally separated rats
NMS CCL	Non maternally separated + CCL rats
O_2	Oxygen
OD	Optical density
OFT	Open field test
Orexin A	Orexin peptide A
Orexin B	Orexin peptide B
ORL1	Opioid receptor-like
OXR-1	Orexin receptor 1
OXR-2	Orexin receptor 2
PBS-T	Phosphate buffered saline with Tween
PFC	Prefrontal cortex
PND	Postnatal day
PNPP	p-nitrophenyl phosphate
PVDF	Polyvinylidene difluoride
PVN	Paraventricular nucleus
RAG	Rabbit anti goat
RHT	Retino hypothalamic tract
rpm	Rotations per minute
SAD	Seasonal affective disorder
SCN	Suprachiasmatic nucleus
SD	Sprague Dawley
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error mean

SNRIs	Selective norepinephrine reuptake inhibitors
SSRIs	Selective serotonin reuptake inhibitors
TIFF	Tag image file format
UCMS	Unpredictable chronic mild stressors
UK	United Kingdom
USA	United States of America
V	Volts
VTA	Ventral tegmental area

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ABSTRACT

Background: Depression is a debilitating mood disorder, negatively affecting an individual's health and well-being. Despite this, the aetiology of depression remains poorly understood. Consistently, depression treatments are far from satisfactory due to limited efficacy and adverse side effects often associated with them, suggesting a need to improve the current animal models of depression in order to understand the basic mechanisms of the disorder. In an attempt to elucidate the pathophysiology of depression, a rodent model of depression (maternal separation, MS) is used to study the neurobiological mechanisms implicated in depression. However, MS alone produces inconsistent findings and often additional stressors are used to exaggerate the effects of MS. To create a more robust model of MS, MS rats were exposed to chronic constant light (CCL). However, contradictory findings have been reported with CCL. **Aims:** This study aimed to explore the effects of additional CCL in an MS model by measuring glutamate and potassium-stimulated [^{3}H]DA release in the nucleus accumbens (NAc), testing the effects of CCL on serotonin (5-HT) levels in the hypothalamus and prefrontal cortex (PFC) and measuring μ-opioid receptor (MOR-1) levels in the NAc and orexin receptor (OXR-1 and OXR-2) levels in the PFC. **Methods:** In order to achieve these aims four experimental groups were chosen, out of which two groups; non-maternally separated (NMS) rats and maternally separated (MS) rats were exposed to CCL for 3 weeks during adolescence and the remaining two groups; NMS and MS rats were not subjected to CCL. At postnatal day 80 (adulthood), rats were decapitated and brain tissue collected for analysis of glutamate- and potassium-stimulated [^{3}H]DA release in the NAc using in vitro superfusion. Serotonin levels in the hypothalamus and PFC were determined using Enzyme-Linked ImmunoSorbent Assay (ELISA). Western blot analysis was used to measure MOR-1 levels in the NAc, OXR-1 and OXR-2 in the PFC. **Results:** MS caused a significant decrease in glutamate-stimulated [^{3}H]DA release in the NAc. In the NAc shell, CCL exposure revealed a trend towards a decrease in [^{3}H]DA release in response to both glutamate- and potassium-stimulation. Moreover, in the hypothalamus NMS and MS rats subjected to CCL had significantly increased 5-HT levels compared to NMS and MS rats without

CCL exposure. In the PFC CCL had a significant effect on 5-HT levels and it was revealed that NMS CCL rats had decreased 5-HT levels compared to NMS rats. Similarly, MS CCL rats had significantly decreased 5-HT levels compared to NMS. MS and CCL did not have any significant effect on MOR-1 protein levels in the NAc. On the other hand, MS rats had increased OXR-1 and OXR-2 proteins levels in the PFC compared to NMS and MS CCL rats. **Conclusion:** MS decreased glutamate-stimulated [^{3}H]DA release in the NAc. Serotonin levels in the hypothalamus and PFC were altered by the effects of MS and CCL. Furthermore, MS exposure increased OXR-1 and OXR-2 protein levels in the PFC. However, MS and CCL did not alter MOR-1 protein levels in the NAc. Therefore, this study has demonstrated that CCL exaggerated the effects of MS and created a more robust model of MS.

CHAPTER 1: INTRODUCTION

1.1 Definition and characteristics of major depressive disorder (MDD)

Major depressive disorder (MDD) is characterised by a prolonged depressed mood or anhedonia (a loss of interest and pleasure in partaking in daily activities such as occupational and educational activities) which often persists for a minimum of 2 weeks (American Psychiatric Association, 2013). During this period, depressed individuals often experience additional symptoms such as changes in appetite, weight and sleep patterns, further interfering with the individual's life style and well-being (American Psychiatric Association, 2013). Moreover, fatigue, loss of energy, poor concentration, indecisiveness, suicidal thoughts, feelings of worthlessness and guilt are also reported in depressed individuals (American Psychiatric Association, 2013). Depression affects both women and men; however women are more prone to developing depression than men (Rubinow et al., 1998; Cochran and Rabinowitz, 2000; American Psychiatric Association, 2013).

1.2 Prevalence of depression

Depression is one of the most prevalent psychiatric disorders affecting approximately 10- 15% of the population at some point in their lives (World Health Organisation, 1996). It is considered to be more detrimental to health than chronic diseases like diabetes and arthritis, which is indicative of the burden this disorder has on health (Moussavi et al., 2007). Furthermore, depression is proposed to be the fourth major cause of disability globally and is believed to be the highest cause of disease burden in the next decade, further providing evidence of the debilitating effects of depression on health (Murray and Lopez, 1997; World Health Organisation, 2010). It is also estimated that about 9 million people are diagnosed annually with depression in the USA alone (Kessler et al., 2005). These high levels of depression have profound cost implications due to lost productivity as well as treatment expenses (Kessler et al., 2005; Kessler, 2012). For example, $83.1

billion and £9 billion are spent in the USA and UK respectively on treating depression (Kessler et al., 2005; Kessler, 2012). Although, the pravalance of depression is low in South Africa (9.7 %) compared to the USA (21.4 %), cases of lost productivity have also been documented, indicating the severity of depression on health (Tomlinson et al., 2009).

Despite the detrimental effects of depression on economy, health and lifestyle, the aetiology and pathology of depression is still not fully understood as depression is a result of complex interactions involving genetic, epigenetic, biochemical, environmental and psychosocial factors (Billings et al., 1983; Brown and Anderson, 1991; Judd et al., 1996; Lupien et al., 2009). However, attempts have been made to treat depressed individuals using several treatment strategies which include antidepressant drugs, psychotherapy, electroconvulsive therapy (ECT), brain stimulating therapies and light therapy (Rosenthal et al., 1984; Kripke et al., 1992; Rohan et al., 2004; Lisanby et al., 2007; Ashkenazy et al., 2009).

1.3 Treatment of depression

Earlier studies considered depression to be caused by deficient monoamine levels such as serotonin (5-HT), norepinephrine (NE) or even dopamine (DA) at specific receptor sites (Schildkraut, 1965). When this hypothesis was introduced it led to an important fundamental principle in understanding the neuro-pathophysiology of depression (Mao, 2011; Willner et al., 2013). However, this hypothesis has been further refined since the 1960s as more evidence has been accumulated over the years. Further research suggests that other biological systems (e.g. abnormal circadian rhythms) other than the monoamine systems are also implicated in the pathophysiology of depression (Jindal and Thase, 2004; Fuchs et al., 2006; Marazzotti et al., 2009; Willner et al., 2013). Despite this, the monoamine depression hypothesis remains a central research area aiding in the development of new medication for treating depression (Willner et al., 2013).

Antidepressant drugs are one of the most widely used treatments for depression (Danese and Pariente, 2008). Antidepressants' mode of action is to enhance

levels of 5-HT, NE and DA and, increase their neurotransmission at receptor sites by blocking their reuptake into presynaptic terminals (Khwawam et al., 2006; Denese and Pariente, 2008; Willner et al., 2013). Initial medication included tricyclic compounds such as imipramine and monoamine oxidase inhibitors, iproniazids which also reverse depressive symptoms by increasing 5-HT, NE and DA levels through inhibition of their re-uptake transporters and enzymes that break down 5-HT, NE and DA monoamines (Khwawam et al., 2006; Denese and Pariente, 2008; Willner et al., 2013). Despite the high efficacy of these drugs in treating depression, they are no longer popular as they induce unwanted side effects such as heart problems, dizziness, drowsiness, dry mouth and weight gain (Nestler, 1998; Khwawam et al., 2006; Denese and Pariente, 2008). As a result, newer antidepressants are commonly used since they are better tolerated and have fewer side effects than older antidepressants (Monteleone et al., 1995; Papp et al., 2003; Khwawam et al., 2006; Kennedy et al., 2006; Sanz and Arranz, 2008).

Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, sertraline and escitalopram are the most commonly used antidepressants. Serotonin and norepinephrine reuptake inhibitors (SNRIs) consisting of venlafaxine, sibutramine and duloxetine, are similar to SSRIs and also form part of newer generation antidepressants (Khawam et al., 2006; Denese and Pariente, 2008; Lanfumey et al., 2013). SSRIs selectivity inhibit the reuptake of only 5-HT whereas the SNRIs inhibits both 5-HT and NE reuptake transporters thereby leading to increased 5-HT and NE levels in the synaptic cleft. Antidepressants used to enhance DA neurotransmission (e.g. bupropion) are also effective in relieving depressive symptomology (Chouinard, 1983; Ferris and Cooper, 1993).

SSRIs and SNRIs have also been reported to produce headaches, nausea, insomnia, and sexual problems and even increase the risk of seizures, suggesting that they are far from satisfactory due to these adverse side effects (Khwawam et al., 2006; Ginsberg, 2009; Fava and Ffidan, 2011). Despite the replacement of older antidepressants by newer ones, antidepressants continue to have delayed onset of action and variable response rates (Khwawam et al., 2006; Ginsberg, 2009; Holtzheimer and Mayberg, 2011). For example, a large percentage of patients do not respond to antidepressant drug and of those who do respond a

large percentage tend to relapse even if the medication is continued for 6 months to a year following behavioural recovery (Trivedi et al., 2006; Sartorius et al., 2007; Holtzheimer and Mayberg, 2011).

Depressed individuals are also provided with strategies and solutions to assist in coping with stress and negative thoughts in an attempt to manage the disorder and to allow them to resume their daily activities (American Psychiatric Association, 2000). This form of treatment is called psychotherapy and is made up of two types of therapies, namely cognitive behavioural therapy (CBT) and interpersonal therapy (IPT) (Reynolds et al., 1999; American Psychiatric Association, 2000; Rohan et al., 2004). CBT assists individuals by reconstructing their negative thoughts so that they can identify the problems that are causing depression thereby preventing the disorder from progressing (American Psychiatric Association, 2000). Similarly, IPT also helps depressed individuals to deal with their troubled relationships by finding solutions to manage depression and preventing it from progressing (Reynolds et al., 1999; American Psychiatric Association, 2000). Psychotherapy can also be used in combination with antidepressants in cases where depression is severe (American Psychiatric Association, 2000).

Brain stimulating therapies are used in cases where patients are resistant to pharmacological and behavioural treatments (American Psychiatric Association, 2000; Rohan et al., 2004; Linsanby, 2007). Examples include ECT, vagus nerve stimulation and repetitive transcranial magnetic stimulation (Rohan et al., 2004; Linsanby, 2007). Brain stimulating therapies involve the application of electrical impulses, magnets or implants on the head in order to amplify neurotransmission at synapses, especially in brain areas implicated in depression (American Psychiatric Association, 2000; Rohan et al., 2004; Linsanby, 2007). For example, ECT is the oldest and most widely used stimulation therapy. This technique is performed by placing electrodes on certain areas of the head that enable electrical current to flow through the brain (APA American Psychiatric Association, 2000, Rohan et al., 2004; Linsanby, 2007). ECT is usually administered 3 times in a week until depressive symptoms are alleviated.

Sleep deprivation and light therapy are gaining more attention in the medical research field, not only as a treatment for depression but also as a model to study the pathophysiology of depression (Rosenthal et al., 1984; Kripke et al., 1992; Rohan et al., 2004; Gorgulu and Caliyurt, 2009; Dimatelis et al., 2012; Hines et al., 2013). Sleep deprivation has been shown to have a high success rate (approximately 60%) in relieving symptoms of depression; however this effect is temporary, lasting for few hours or a month if patients are also treated with antidepressant drugs (Wu and Bunney, 1990; Gorgulu and Caliyurt, 2009). Furthermore, sleep deprivation has been positively correlated with increased serum brain-derived neurotropic factor (BDNF), which is often decreased in depressed patients (Haung and Reichardt, 2001; Feng et al., 2003; Karege et al., 2005; Gorgulu and Caliyurt, 2009). Similarly, 12 hours of sleep deprivation improved depressive-like symptoms in C57BI6 mice strain, known to exhibit high depressive-like behaviours (Hines et al., 2013).

One of the earliest studies to explore the effects of light therapy on depression was conducted by Rosenthal et al. (1984). Subsequently, other studies have also used light therapy to treat depression associated with seasonal affective disorder (SAD) (Ashkenazy et al., 2009; Rosenthal et al., 1984; Kripke et al., 1992; Rohan et al., 2004). SAD is believed to be caused by disrupted circadian rhythms due to shorter light and longer dark periods during autumn and winter seasons (Ashkenazy et al., 2009; Rosenthal et al., 1984; Kripke et al., 1992; Rohan et al., 2004). As a result, the main function of light therapy is to restore the body's normal circadian rhythms and biological systems such as the opioid receptor system, in particular the μ-opioid receptor (MOR) (Kripke et al., 1992; Rohan et al., 2004; Ashkenazy et al., 2009; Dimatelis et al., 2012). For example, Dimatelis et al. (2012) showed that reduced MOR levels in a rodent model of depression, maternal separation (MS), can be reversed by treatment with continuous lighting.

Despite the improvements in treatments of depression and knowledge about the underlying mechanisms of depression, the aetiology of depression is still not fully understood. This suggests that new studies are required to elucidate the pathophysiology of depression in order to provide more effective treatment. Studies aimed at understanding the factors that contribute to the development of

depression are crucial for providing further insight into the pathophysiology of depression.

1.4 Early life stressors are risk factors of depression

Epidemiological studies indicate that depression is caused by many internal and external factors that include genetic, environmental and psychosocial factors (Billings et al., 1983; Brown et al., 1986; Kendler et al., 1995; Judd et al., 1996; Barr et al., 2008). For example, an individual exposed to internal factors such as genetic variants associated with depression is at increased risk of developing depression, more especially if the same individual is subjected to external factors such as early life stress (e.g. childhood abuse) (Figure 1.1). Exposure to early life stress during the postnatal period when the brain is still developing might alter the stress system, the hypothalamic-pituitary-adrenal (HPA) axis; thereby increasing the risk of developing depression later in life (Lupien et al., 2000; Geoffroy et al., 2006; Albers et al., 2008; Lupien et al., 2009). Therefore, exposure to both internal and external factors including a dysregulated HPA axis activity further increases the risk of triggering a depressive episode, more especially if the same individual is subjected to additional stressors during adulthood (Albers et al., 2008; Lupien et al., 2009) (Figure 1.1).

It is proposed that psychosocial factors, in particular early stressful life events are one of the most effective factors to induce depressive episodes (Kendler et al., 1999). Early adverse life stressors or traumatic experiences do not only predispose individuals to depression but also to a number of neuropsychiatric disorders (Robertson and Bowlby, 1952; Kendler et al., 2005; Heim et al., 2000, 2020). Moreover, children subjected to physical, emotional or sexual abuse are highly susceptible to mood disorders such as anxiety and depression in adulthood (Gibb et al., 2007; Felitti et al., 1998). It is believed that the development of depression is caused by a dysregulated HPA axis activity due to early life stress exposure (Young et al., 2006; Brent et al., 2009).

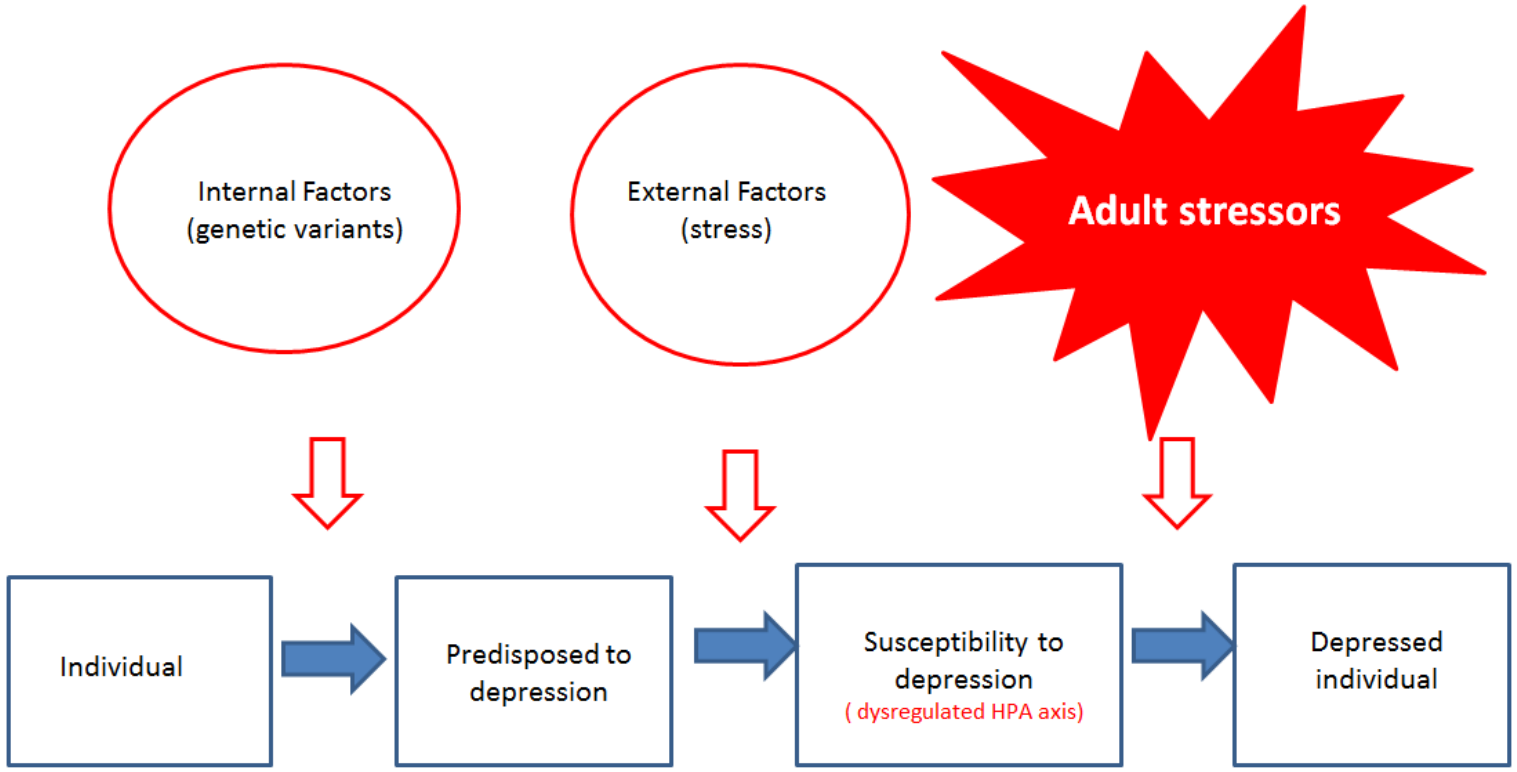


Figure 1.1: Risk factors of depression. A schematic diagram showing the multifactorial relationship between internal (genetic variants) and external factors (early life stress e.g. childhood abuse) in the development of depression. Altered hypothalamic-pituitary-adrenal (HPA) axis activity as a result of internal and external factors further increases the risk of developing depression, especially if the individual is subjected to additional stressors such as a loss of a job during adulthood. This might trigger a depressive episode leading to depression (Billigns et al., 1983; Brown et al., 1986; Lupien et al., 2000; Matthew et al., 2005; Geoffroy et al., 2006; Albers et al., 2008; Lupien et al., 2009).

1.5 The stress system: HPA axis

The HPA axis is considered an important system in mediating stress responses (Lupien et al., 2009). During stress, the corticotropin-releasing factor (CRF) hormone of the hypothalamus paraventricular nucleus (PVN) binds to the receptors of the anterior pituitary gland to stimulate the release of adrenocorticotropic hormone (ACTH) (Figure 1.2) (Lupien et al., 2009). ACTH in turn activates the secretion of glucocorticoids (cortisol in humans and corticosterone (CORT) in rodents) from the adrenal cortex into the bloodstream (Lupien et al., 2009). In order to inhibit further secretion of CORT, the rising circulating cortisol exerts a negative feedback inhibition to prevent further release of CORT once the stress has passed (Lupien et al., 2009). Negative feedback inhibition is mediated through two types of receptors, namely mineralocorticoid (MR) and glucocorticoid receptor (GR) (Dallman et al., 1987; de Kloet et al., 1997; Lupien et al., 2009). These receptors are expressed in many parts of the central nervous system (CNS), in particular in brain areas implicated in the stress response such as the hippocampus, amygdala and PFC. These areas are important for mediating negative feedback by glucocorticoids on the HPA axis in response to stress (de Kloet et al., 1997). The ability of MR and GR receptors to suppress the HPA axis activity in response to stress plays an important role in enabling an individual to cope or adapt to stress (de Kloet et al., 1997).

It is important to note that the severity, chronicity, predictability, coping ability and previous exposure to stressors are also factors that influence the way in which individuals respond to stressors (Paykel, 2001; Kendler et al., 1999). It is the inappropriate response to stress that often leads to increased susceptibility and development of psychiatric disorders, including mood disorders (Figure 1.2) (Holsboer et al., 1986; Menteleone et al., 1995; de Kloet et al., 1997; Lupien et al., 2009). The effects of early life stress are often demonstrated by increased HPA axis activity, which is considered a hallmark characteristic of depression (Nemeroff et al., 1984; Holsboer et al., 1986; Menteleone et al., 1995).

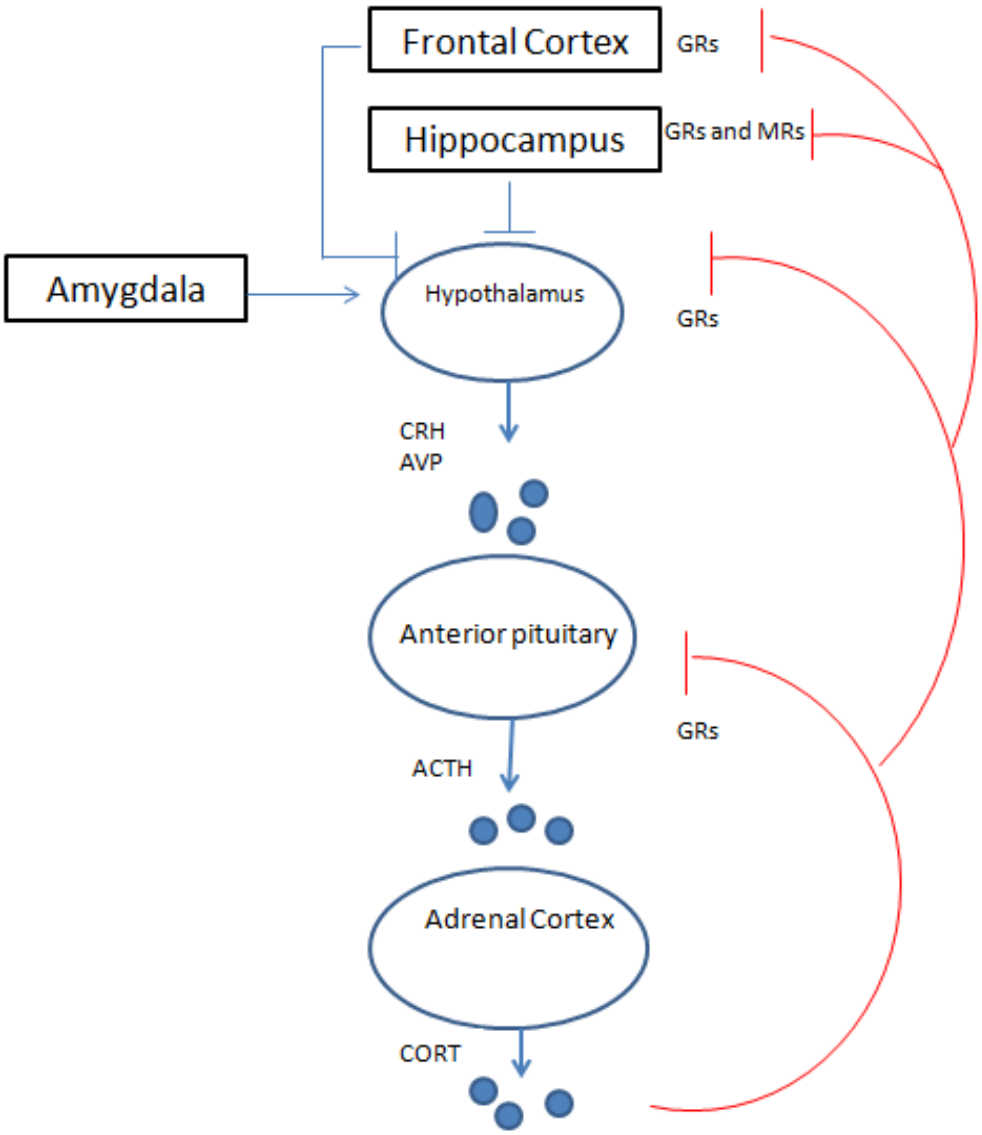


Figure 1.2: The stress system, HPA axis. During a stressful event the HPA axis responds by secreting corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus. The CRH and AVP activate the anterior pituitary gland thereby releasing adrenocorticotropic hormone (ACTH) which in turn causes production of glucocorticoids such as cortisol and corticosterone (CORT). Increased CORT levels cause negative feedback to inhibit further CORT release by activating glucocorticoid receptors (mineralocorticoid (MR) and glucocorticoid receptor (GR) which are located in the pituitary gland, hypothalamus, hippocampus and frontal cortex. This in turn suppresses HPA axis activity leading to decreased production of glucocorticoids thereby maintaining homeostasis. The amygdala is also involved in the regulation of the stress response by activating the HPA axis in particular in coping with the fear responses of stress (Lupien et al., 2009).

Increased HPA axis activity is indicated by higher basal plasma cortisol, ACTH levels and hyper secretion of CRF, suggesting inability to cope with stress (Pfeffer et al., 2007; Holsboer et al., 1986; Menteleone et al., 1995). Prolonged exposure to high levels of glucocorticoids impairs the negative feedback mechanism responsible for decreasing the HPA axis activity (Pfeffer et al., 2007; Holsboer et al., 1986; Menteleone et al., 1995). For example, women who were abused as children displayed increased HPA axis activity in response to a standard laboratory stressor when matched to non-abused women (Heim et al., 2000). Moreover, those with a history of childhood abuse and a recent major depressive episode were reported to have more than six fold greater ACTH response to a stress compared to controls (Heim et al., 2000).

The relationship between a parent and a child as well as the psychological state of the mother has a potent effect on the activity of the HPA axis of the child (Lupien et al., 2009). For example, children whose mothers experienced stress, depression and anxiety during pregnancy were reported to have increased basal HPA axis activity at 6 months, 5 years and 10 years of age (Lyons-Ruth et al., 2000; O'Connor et al., 2005). Similarly, children whose mothers were depressed during their early life were susceptible to developing enhanced HPA axis activity and depression during adolescence (Lupien et al., 2000). Furthermore, children that spent a significant amount of time away from their mothers as toddlers in day care centres were reported to have altered glucocorticoid levels (Geoffroy et al., 2006). This means that exposure to stress resulting from child-mother interaction can alter the HPA axis activity and increase the risk of experiencing stress or mood disorders in later life (Heim et al., 2000; Albers et al., 2008; Lupien et al., 2009).

Based on these findings it is evident that early life stressors play a significant role in the development of mood disorders, in particular depression. It is important to note that childhood represents a critical point in time in which the effects of stress exposure can predispose children to developing depression later in life (Albers et al., 2008; Lupien et al., 2009). This is because brain areas such as the amygdala, hippocampus and frontal cortex which are involved in the regulation of the HPA axis in response to stress are still maturing and are most vulnerable to the effects

of stress during this stage of development (Figure 1.3) (Lupien et al., 2000; Geoffroy et al., 2006; Albers et al., 2008; Lupien et al., 2009).

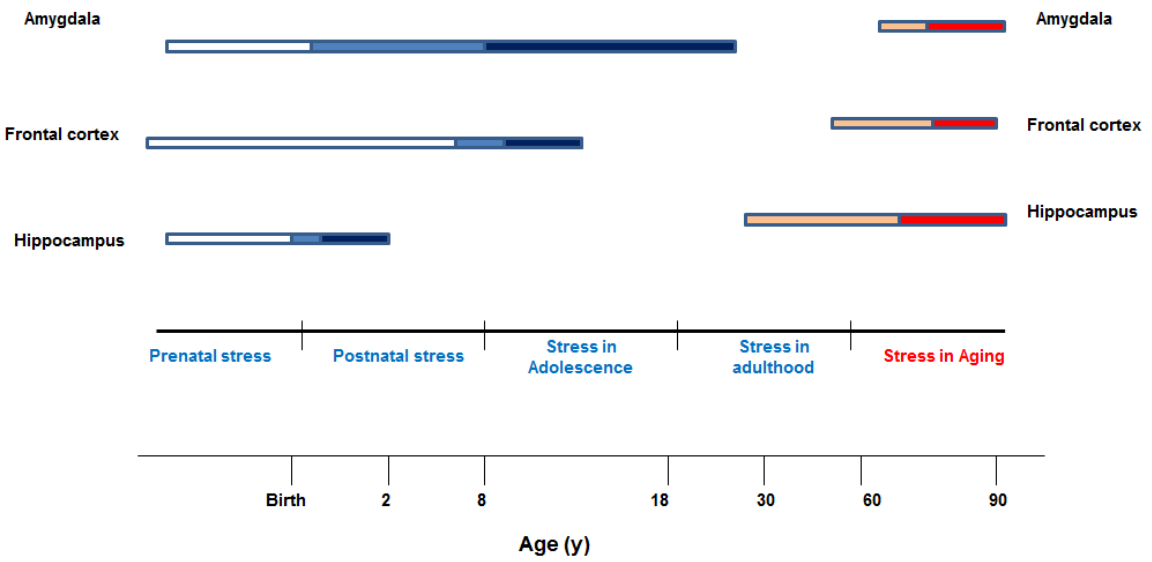


Figure 1.3: The life cycle model of stress. The impact of stress exposure at different stages of life during the development and declining stages of the human brain areas (amygdala, frontal cortex and hippocampus) involved in the regulation of the HPA axis activity. From birth to 2 years, the hippocampus matures first (represented by the navy colour segment of the bar line), followed by the frontal cortex which is fully developed by adolescence. Amygdala is the last brain area to mature; its volume only matures in adulthood specifically in the late 20s. Despite this, the amygdala is less vulnerable to the effects of stress than the frontal cortex since at 2 years of age this area is more developed than the frontal cortex which only starts to mature around 8 years of age. In adulthood and aging (orange and red bars represent the brain areas undergoing the most rapid decline as a result of aging). The hippocampus is the first brain area to undergo a rapid decline as a result it is most vulnerable to the effects of stress, followed by the frontal cortex and amygdala (Lupien et al., 2009).

Depression continues to affect millions of people around the world and the fact that this disease is a major burden on the economy, health and lifestyle makes it a serious health concern that needs to be addressed (Murray and Lopez, 1997; World Health Organisation, 2010; Kessler, 2012). Hence, it is important to understand the underlying mechanisms of depression. Therefore, it is reasonable to propose an animal model that mimics the effects of early life stress such as the

MS as an ideal model to elucidate the biological mechanisms implicated in depression. Not only are animal models useful in elucidating the basic mechanisms of the disorder, they are also important in the development of novel and more effective therapeutic targets for treating depression (Geyer and Markou, 1995; Kendler et al., 1999; Marais et al., 2008; Abelaira et al., 2013).

1.6 MS as a model of early life stress

An animal model must adhere to specific criteria in order to model certain aspects of the human condition; these criteria include face, construct and predictive validity (Table 1.1) (Geyer and Markou, 1995; Abelaira et al., 2013). Face validity refers to the similarities between the behaviours exhibited by the animal model and those often observed in the human condition (Geyer and Markou, 1995; Abelaira et al., 2013). Construct validity requires the pathology or neurochemical processes displayed in the animal model to be similar to that of a human condition (Geyer and Markou, 1995; Abelaira et al., 2013). Predictive validity requires behavioural changes in the animal model after treatment to predict similar changes in the human condition (Geyer and Markou, 1995; Abelaira et al., 2013). Since early life stressors or traumatic experiences during childhood are considered the most potent factors to induce neuropsychiatric disorders in particular depression and anxiety disorders later in life (Kendler et al., 1999; Paykel 2001; Pryce et al., 2005; Geoffroy et al., 2006; Albers et al., 2008; Heim et al., 2010 Abelaira et al., 2013), this study has chosen MS as an animal model to study the effects of early adverse experiences on the brain. The aim of this study was to increase our understanding of the pathophysiology of depression. The use of MS as a model of early life stress is further supported by the findings that children subjected to early life stress exhibit altered HPA axis activity (Albers et al., 2008; Geoffroy et al., 2006).

Table 1.1: Criteria of an animal model for a human disorder

Criteria	Description	Example
Face validity	Similarities between behaviour exhibited by the animal model and the specific symptoms of the human disorder	Increased immobility time in the FST or decreased sucrose intake (anhedonia) in animals should correspond to depressive symptoms in humans (Ladd et al., 2000; Daniels et al., 2004; Dimatelis et al., 2012).
Construct validity	Similarities in the pathophysiology or neurochemical processes of the disorder between the animal model and the human disorder	Dysregulated HPA axis, increased ACTH or CORT levels should be observed in both depressed animal and human subjects (Aisa et al., 2007; Marais et al., 2008).
Predictive validity	States that one should be able to predict changes in the human condition based upon the changes in the animal model. That is, improvement or reduction of depressive symptoms observed after treatment in an animal should also be observed in a human condition.	Administration of an antidepressant drug in the animal should ameliorate depressive-like symptoms (Di Matteo et al., 2001; Dremencov et al., 2004).

Adapted from (Geyer and Markou, 1995; Abelaira et al., 2013)

Abbreviation: Adrenocorticotropic hormone (ACTH), corticosterone (CORT), forced swim test (FST), hypothalamus-pituitary-adrenal (HPA) axis

1.7 Maternal separation (MS)

MS and early life deprivation are the two most used animal models to study the effects of postnatal stress (Aisa et al., 2007; Marais et al., 2008; Zimmerberg et al., 2009; Dimatelis et al., 2012). Both of these paradigms refer to the separation of pups from their mothers (dam) for a short period of time. In an MS model, the pups are kept together as a litter as opposed to the early life deprivation model in which pups are isolated from the dam and from the rest of the litter (McCormick et al., 1998; Zimmerberg et al., 2009). One of the earliest studies to investigate the effects of MS on behaviour was conducted on infant monkeys (Hinde et al., 1966; Hrdina et al., 1979). In this study, separation of the infant monkeys from their mothers resulted in behaviour resembling despair in humans (Hinde et al., 1966; Hrdina et al., 1979).

Similarly in rats, separation of pups during the first 3 weeks of life is stressful since the pups are completely dependent on their mothers during this period (Slotten et al., 2006; Aisa et al., 2007; Marais et al., 2008). MS can be performed for a short period (1 hour) or for prolonged periods (3-6 hours) during the 1-3 weeks after birth (Hout et al., 2002; Marais et al., 2008; Wilber and Wellmar, 2009). This model causes increased basal CORT levels, increased CRF and lower adrenal weight in separated rats compared to non-separated rats, indicating that HPA axis activity had been altered (Slotten et al., 2006; Aisa et al., 2007; Marais et al., 2008).

Changes in HPA axis activity of MS animals are associated with anxiety-like behaviours in the elevated plus maze (EPM) and open field test (OFT) and depressive-like behaviours in the FST (Ladd et al., 2000; Daniels et al., 2004; EL Khoury et al., 2006; Marais et al., 2008). Similarly, rats exposed to 3 hours daily of MS over 3 weeks from postnatal day (PND); PND2 - 21 spent less time in the open arm of the EPM, indicating that animals subjected to MS stress display anxiety-like behaviours (Aisa et al., 2007). Similarly, 2 weeks of MS (P2-P14) induced anxiety-like behaviours in the EPM (Hout et al., 2001; Lee et al., 2007). MS rats also have decreased preference for sucrose solution, which is indicative of anhedonic-like behaviours as found in depression (Aisa et al., 2007; Hout et al.,

2001). Typically, rats prefer sweetened fluids (sucrose) over water; however this preference is reduced after MS (Aisa et al., 2007).

Due to the altered HPA axis activity, MS rats are further predisposed to reacting to additional stressors such as behavioural tests (FST, OFT, EPM) and restraint stress that potentiate the effects of MS causing depressive- and anxiety-like behaviours in adulthood (Plotsky and Meaney, 1993; Veenema et al., 2006; Lee et al., 2007; Marais et al., 2008). Similarly, early life stress when combined with subsequent adult stress has been reported to increase the risk of mood disorders, specifically depression and anxiety disorders in human studies (Heim et al., 2000; Heim et al., 2008). These studies suggest that exposure to additional stressors in animals already subjected to MS stress might be useful in providing further insight into the aetiology of depression.

1.8 Chronic constant light (CCL) as an additional stressor

Over the years it has become evident that depression is not only due to deficient monoamine levels as had previously been proposed by Schildkraut (1965). Other biological systems such as the circadian rhythms are also implicated in the pathophysiology of depression (Jindal and Thase, 2004; Fuchs et al., 2006; Willner et al., 2013). Moreover, the fact that sleep disturbance (insomnia or hypersomnia), is one of the most prominent features of depression leading to a loss of concentration and decreased energy levels, suggests that abnormal circadian rhythms may be implicated in the pathophysiology of depression (Salomon et al., 2003; Cao and Guilleminault, 2011).

Circadian rhythms are defined as biological processes that have endogenous oscillations with a period of approximately 24 hours (Bailey and Silver, 2014). Under normal conditions, the circadian rhythms are synchronised by external stimuli called zeitgebers, also known as time givers (Krishnan and Neslter, 2011; Bailey and Silver, 2014). The 24 hour light/dark (L/D) cycle is the main entraining zeitgeber in the CNS, in particular for the suprachiasmatic nucleus (SCN) of the hypothalamus (Dubocovich, 2007; Krishnan and Neslter, 2011; Catena-Dell'Osso

et al., 2012; Bailey and Silver, 2014). The SCN is synchronised by light via signalling through the retino hypothalamic tract, one of the three pathways that send information to the SCN (Dubocovich, 2007; Krishnan and Neslter, 2011; Catena-Dell'Osso et al., 2012; Bailey and Silver, 2014) (Figure 1.4). The other two pathways that also send input to the SCN include the geniculo hypothalamic tract and the serotonergic (5HT-ergic) systems (including both the dorsal raphe, (DRN) and medial raphe nuclei, (MRN) (Dubocovich, 2007; Catena-Dell'Osso et al., 2012; Krishnan and Neslter, 2011; Bailey and Silver, 2014). The retino hypothalamic tract sends photic information from the non-image forming photoreceptors known as photosensitive retinal ganglion cells (Provencio et al., 2000; Berson net al., 2002; Shirani and St Louis, 2009) (Figure 1.8.1). These cells contain a photo sensitive pigment, melanopsin, which is important in transmitting light synchronising information into neural impulses to be used by the SCN (Berson et al., 2002; Moore and Speh, 2004). Similarly, the geniculo hypothalamic tract pathway via the intergeniculate leaflet (which in turn receives input from the orexin system) also sends photic input to the SCN about the L/D cycle and level of arousal (Mintz et al., 2001). The 5HT-ergic neurons that originate from the MRN and DRN inhibit SCN activity in response to light (Berson et al., 2002; Moore and Speh, 2004) (Figure 1.4).

The main function of the SCN is to generate circadian rhythms and prepare the individual to adapt to the daily changes in the environment (Wirz-Justice, 2006). Some of the many biological functions that exhibit circadian rhythms include body temperature, heart rate, melatonin, cortisol and growth hormone secretion (Ritcher et al., 2004; Mazzolli et al., 2011). The SCN is also known to regulate the circadian rhythm of glucocorticoid levels; as a result lesions of the SCN disrupt the secretion of CORT (Campbell and Dawson, 1990; Redlin, 2001). In both nocturnal (rodents) and diurnal species (humans) an increase in glucocorticoid levels is associated with arousal. In diurnal species glucocorticoid levels peak in the early hours of the morning, while the opposite is found in nocturnal species (Campbell and Dawson, 1990; Redlin, 2001). These findings indicate that the SCN plays an important role in regulating the circadian rhythms of the stress system (Bao et al., 2008).

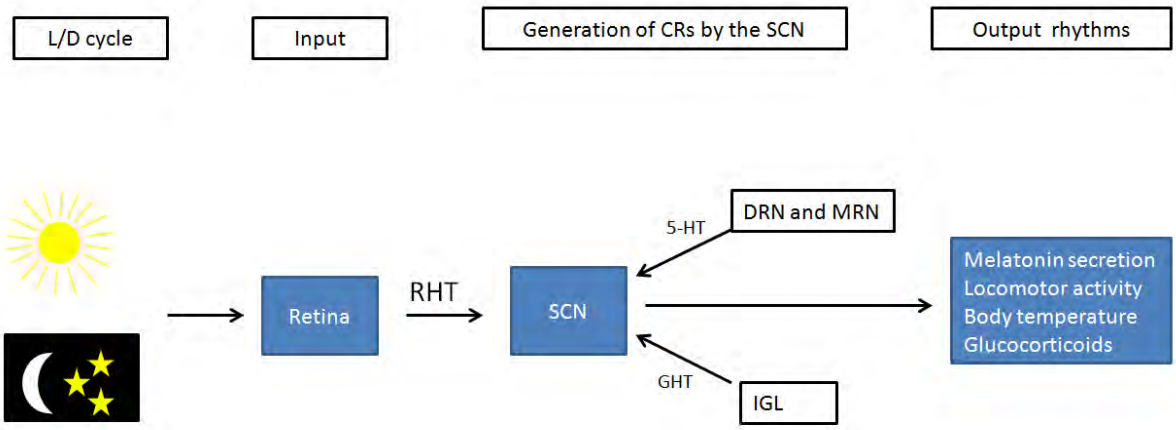


Figure 1.4: Generation of circadian rhythms for biological functions. The 24 hour light/dark (L/D) cycle synchronises the suprachiasmatic nuclei (SCN) via the retino hypothalamic tract (RHT). The SCN also receives input from the 5HT-ergic neurons that originate from the dorsal and median raphe (DRN and MRN) and from the geniculo hypothalamic tract (GHT) which also receives photic and arousal input via the intergeniculate leaflet (IGL). Inputs to the SCN lead to the generation of circadian rhythms of many important biological functions such as melatonin secretion, locomotor activity and glucocorticoid release. Disturbances in this system predispose individuals to developing depression (Campbell and Dawson, 1990; Redlin, 2001; Krishnan and Neslter, 2011).

The AVP neurons of the SCN regulate CRH neurons of the PVN by inhibiting their activity during stress (Kalsbeek et al., 1992). However, increased cortisol levels decrease AVP activity resulting in failure to inhibit CRH neurons (Liu et al., 2006; Kalsbeek et al., 1992). This in turn causes the CRH neurons to activate the anterior pituitary to release ACTH and subsequently releasing more cortisol (Kalsbeek et al., 1992). This might explain the decreased SCN activity due to increased plasma cortisol levels; suggesting that the interaction of the CRH-AVP neurons is crucial for controlling glucocorticoid release in response to stress (Kalsbeek et al., 1992; Liu et al., 2006). Therefore, MS exposure might decrease SCN activity and predispose rats to additional stress (CCL) in adulthood. CCL exposure might also exacerbate the effects of MS by further reducing SCN activity, thus increasing the risk of developing depression.

Since melatonin secretion is regulated by both the L/D cycle and exposure to stress, processes that control SCN activity, suggests that melatonin production might be susceptible to the effects of MS and CCL (Rieter et al., 2000). Functionally, melatonin translates photoperiodic information by activating melatonin receptors (MT1 and MT2) located in the SCN to regulate many physiological functions that vary with season including reproduction, appetite and sleep (Rieter et al., 2000; Hazlerigg and Wagner, 2006). MT1 inhibits firing of neurons in the SCN and is believed to be involved in promoting sleep (Rieter et al., 2000). On the other hand, MT2 is responsible for the entrainment of circadian rhythms (Hatter et al., 2003). During the dark, the SCN transmits photic input to the pineal gland to stimulate pinealocytes to synthesize and release melatonin (Campbell and Dawson 1990; Redlin, 2001; Simonneaux & Ribelayga, 2003; Catena-Dell 'Osso et al., 2012). Therefore, darkness activates melatonin synthesis whereas light inhibits melatonin production (Monteleone et al., 2011). Melatonin secretion is greater at night in both diurnal and nocturnal species (Campbell and Dawson, 1990; Redlin, 2001; Claustrat et al., 2005). However, in diurnal species melatonin corresponds with decreased activity (beginning of sleep in humans) and in nocturnal species melatonin production is associated with increased activity (active period in rats) (Nagtegaal et al., 1998).

Impaired melatonin activity, whereby melatonin secretion is delayed in depressed patients compared to controls has been reported by Crasson et al. (2004). Furthermore, unipolar and bipolar depressed patients display low levels of melatonin (Beck-Frils et al., 1985; Soutre et al., 1989). The role of melatonin in depression is further supported by the findings that melatonin treatment improves total sleep time and decreases depressive symptoms (Kayumov et al., 2001). These observations indicate that the melatonergic system is necessary for maintaining mood. In accordance with this statement, antidepressant-like effects of melatonin have been reported and adverse effects of chronic stress are reversed by melatonin administration (Kopp et al., 1999). Similarly, agomelatine (an analogue of melatonin) is used for restoring circadian rhythms in individuals with insomnia and jet lag, further supporting the role of melatonin in regulating circadian rhythms (Zisapel, 2001). Agomelatine has a high affinity for 5-HT_{2C} receptors and it is believed that the antidepressant-like effects of agomelatine are

mediated through these receptors (Millan et al., 2003; Papp et al., 2003). Altered 5-HT_{2C} receptor levels have been implicated in depression (Riedel et al., 2002).

Rats, which are nocturnal animals prefer dim light as opposed to bright light (Castelhono-Carlos, 2009). This is also supported by the findings that albino rats adjust better to low light intensity that is below 100 lux than to higher light intensity (Blom et al., 1995). Similarly, rats exposed to 10 days of CCL displayed decreased hypothalamic CRF-like immunoreactivity CRF-L, decreased CR of plasma CORT and increased plasma ACTH (Fischman et al., 1988). Similarly, Mohawk et al. (2007) showed that one hour of light exposure at night resulted in elevated CORT levels with ACTH levels increasing after 15 minutes of exposure. However, light exposure during the day did not result in any changes in glucocorticoid levels. As a result the authors concluded that light exposure during the dark period directly affected the HPA axis leading to the secretion of glucocorticoids, in a manner resembling the secretion of glucocorticoids under a stressful situation. These data, suggest that continuous lighting during the dark acts as a stressor.

It is well known that disruption of the HPA axis also predisposes animals to anxiety and depressive-like behaviours (Plotsky and Meaney, 1993). CCL has been shown to induce anhedonic, anxiety- and depressive-like behaviours (Ma et al., 2007; Martynhak et al., 2011; Tapio-Osorio et al., 2013). Wistar rats that were subjected to 8 weeks of CCL showed a disrupted CR of locomotor activity and plasma melatonin secretion (Tapio-Osorio et al., 2013). CCL also caused increased sucrose intake and anxiety-like behaviours in the OFT as well as elevated levels of CORT (Tapio-Osorio et al., 2013). Furthermore, it was shown that 7 days of continuous light from embryonic day 20 to postnatal day 4 resulted in high anxiety-like behaviours in the OFT as demonstrated by more time spent in the outer zone than in the inner zone of the open field (Roman and Karlsson, 2013). These studies provide substantial evidence that CCL is a stressor; this is demonstrated by the altered HPA axis activity as well as the manifestation of depressive- and anxiety-like behaviours in animals in adulthood (Ma et al., 2007; Martynhak et al., 2011; Tapio-Osorio et al., 2013).

Furthermore, light exposure has also been associated with anhedonic behaviours and manic-like behaviours in rats (Martynhak et al., 2011). For example, 3 weeks of light exposure during adulthood decreased sucrose preference, suggesting anhedonic-like behaviours (Martynhak et al., 2011). However, combination of light exposure during the neonatal period and adulthood reversed the decreased sucrose preference. Light exposure during neonatal period protected rats from developing the anhedonic behaviours caused by additional exposure to light in adulthood (Martynhak et al., 2011). Similarly, light exposure during the neonatal period prevented abnormal circadian rhythms caused by more than 3 weeks of light exposure during adulthood (Cambras et al., 1998; Canal-corretger et al., 2001). Moreover, it was revealed that 12 days of light exposure during the neonatal period was the minimum number of days required to develop a strong circadian rhythm in rats exposed to additional lighting in adulthood (Canal-corretger et al., 2001). Therefore, the strong circadian rhythm as a result of light exposure during the neonatal period protected rats from developing the anhedonic-like behaviours induced by continuous lighting in adulthood (Cambras et al., 1998; Canal-corretger et al., 2001; Martynhak et al., 2011). These findings suggest that rats exposed to continuous lighting only in adulthood are susceptible to developing anhedonic behaviours due to the inability of the circadian rhythms to adjust to the change in photoperiod, providing further evidence to support CCL as an additional stressor and as a suitable model to study depression.

Increased sucrose preference and hyper locomotion phenotypes in animals have been proposed to model the manic spectrum of bipolar disorder (Flaisher-Grinberg et al., 2009a, 2009b). Increased sucrose preference was observed in Black Swiss mice; a mouse strain displaying manic related behaviours such as hyperlocomotion (Flaisher-Grinberg et al., 2009a). Martynhak et al. (2011) also found increased locomotor activity and sucrose preference in rats subjected to 3 weeks of constant light during the neonatal period, suggestive of displaying manic-like behaviours. These findings suggest that the timing of light exposure is an important factor when choosing a stress model, as different behavioural phenotypes are reported to result from exposure to CCL during various developmental stages of the animal. For this study, rats were only subjected to 3 weeks of CCL in adulthood instead of exposure to CCL during the neonatal period,

since light exposure during adulthood leads to anhedonic and depressive-like behaviours. As opposed to light during the neonatal period which results in manic-like behaviours (Ma et al., 2007; Martynhak et al., 2011; Tapio-Osorio et al., 2013).

The abnormal phenotypes induced by CCL suggest that CCL exposure at certain developmental time points might share a similar pathophysiology as bipolar/depressive disorder. Decreased extracellular signal-related kinase (ERK) signalling is often implicated in bipolar disorders and administration of a mood-stabiliser (valproate) reverses the decreased ERK signalling (Hao et al., 2004). Similarly, deficiencies in ERK signalling have also been implicated in the pathophysiology of depression (Haung and Reichardt, 2001; Feng et al., 2003; Karege et al., 2005). ERK plays a major role in regulating the expression of BDNF and reduced ERK is associated with reduced BDNF levels which are often implicated in depression (Haung and Reichardt, 2001; Feng et al., 2003; Karege et al., 2005). Therefore, the presence of anhedonic, anxiety- and depressive-like behaviours after light exposure suggest that CCL is a stressor and that it is implicated in the pathophysiology of depression (Canal-corretger et al., 2001; Ma et al., 2007; Flaisher-Grinberg et al., 2009a; Martynhak et al., 2011; Tapio-Osorio et al., 2013). Hence, CCL was chosen as an additional stressor in order to elucidate the underlying mechanisms implicated in the pathophysiology of depression.

1.9 Dopamine and depression

Dopamine (DA) is a catecholamine and a neurotransmitter that is involved in signalling processes between neurons (Sarkar et al., 2010). DA is produced in the cytoplasm of presynaptic neurons from the amino acid phenylalanine and tyrosine (Dunlop and Nemeroff, 2007). Most of the dopaminergic neurons in the brain are located in brainstem nuclei; retro-rubro field, substantia nigra pars compacta and ventral tegmental area (VTA) (Dunlop and Nemeroff, 2007). DA exerts its effect on postsynaptic neurons through its interactions with one of five subtypes of DA receptors which are divided into two groups (Dunlop and Nemeroff, 2007). The first group is the D1 family which is made up of D1 and D5 receptor subtypes and

the second group, the D2 family consists of D2, D3 and D4 subtypes. In the CNS, circuits containing DA are important regulators of the following functions; motivation, regulating locomotion, cognition, emotion, concentration and ability to experience pleasure (Ikemoto and Panksepp, 1999; McClure et al., 2003; Shirayama and Chaki, 2006; Dunlop and Nemeroff, 2007).

There are three major dopaminergic pathways which innervate various cortical and subcortical structures namely; nigrostriatal, tuberoinfundibular and mesocorticolimbic pathway (Dunlop and Nemeroff, 2007; Shirayama and Chaki, 2006). The nigrostriatal pathway arises from the substantia nigra pars compacta and projects to the dorsal striatum (caudate and putamen) (Dunlop and Nemeroff, 2007). This pathway plays a major role in regulating motor and non-motor functions such as executing and planning movement as well as cognition (Dunlop and Nemeroff, 2007). The tuberoinfundibular pathway originates from the arcuate nucleus of the hypothalamus and projects to the median eminence of the hypothalamus. Functionally, this pathway plays an important role in controlling the release of growth hormone from the anterior pituitary. The mesocorticolimbic pathway is made up of numerous brain areas such as the VTA, ventral striatum (nucleus accumbens, NAc), hippocampus, motor cortex, amygdala and septum. This pathway controls reward-related behaviours, motivation and ability to experience pleasure as well as locomotor activity (Ikemoto and Panksepp, 1999; Shirayama and Chaki, 2006; Dunlop and Nemeroff, 2007).

The mesolimbic dopaminergic system stimulates the NAc, amygdala and septum (Ikemoto and Panksepp, 1999; Shirayama and Chaki, 2006; Dunlop and Nemeroff, 2007). Due to the robust findings of anhedonia in depression, it has been proposed that reduced motivation and decreased energy levels in depressed individuals suggest that the NAc and VTA are implicated in the pathophysiology of depression (Ikemoto and Panksepp, 1999). Of importance is accumbal DA release, as this region regulates important functions such motivation, reward, motor function and learning (Shirayama and Chaki, 2006; Koob and Bloom, 1988; Ikemoto and Panksepp, 1999).

The shell and core are the two subregions of the NAc comprising of the ventral striatum. Dopaminergic activity in the NAc is influenced by many molecules such as glutamate, 5-HT, gamma-aminobutyric acid (GABA), acetylcholine, and neuropeptides. The NAc receives dense glutamatergic innervations from the PFC, amygdala and hippocampus, the same brain regions that are involved in HPA axis regulation in response to stress (Rosso and Nestler, 2013). Therefore, these findings suggest that accumbal DA release is implicated in stress regulation. Indeed, accumbal DA release is highly susceptible to stress and abnormal DA release in the NAc causes anhedonia in both human and animal models of depression (Wilner et al., 1992; Yadid et al., 2001). For example, DA levels are depleted in the NAc of animals experiencing learned helplessness and this can be prevented by administrating a DA agonist (Anisman et al., 1979).

Reports suggest that short-term exposure to various forms of stress enhances accumbal DA release (Kalivas and Duffy, 1995; Tidey and Miczek, 1996). However, long-term exposure to different types of unavoidable stressors decreases DA release in the NAc shell (Di Chiara et al., 1999; Scheggi et al., 2002). For example, mice subjected to unavoidable foot shock showed decreased DA release in the NAc in contrast to mice subjected to escapable foot shock which showed increased DA release in the NAc (Cabib and Pugusi-Allegra, 1994). Similarly, DA reuptake inhibitors were shown to treat depression by increasing DA concentration in NAc (Garattini, 1997). Ichikawa et al. (1998) showed that chronic administration of the antidepressant imipramine and DA agonist, amphetamine, resulted in increased extracellular dopamine levels in the NAc. In a validated genetic model of depression, the Flinders Sensitive Line (FSL) rats showed deficits in 5-HT-induced DA release in the NAC which was restored by antidepressant treatment (Moreo et al., 1996; Ichikawa and Meltzer, 1999; Di Matteo et al., 2001; Zangen et al., 2001; Dremencov et al., 2004).

These findings provide substantial evidence to support the role of DA in depression, in particular accumbal DA release (Wilner et al., 1992; Yadid et al., 2000). Moreover, the fact that accumbal DA interacts with many neurological systems such as the 5-HT, opioid and orexinergic systems justifies the importance of choosing this system to study the pathophysiology of depression (Pecina and

Berridge, 2000; Di Matteo et al., 2001; Dremencov et al., 2004; Baldo and Kelley, 2007; Nocjar et al., 2012). DA release in the NAc after MS and CCL exposure was therefore investigated in an attempt to provide further insight into the pathophysiology of depression. The effects of MS are potentiated by additional stressors such as CCL or restraint stress which impair spatial memory and induce depressive- and anxiety-like behaviours in rodents (Ma et al., 2007; Marais et al., 2008; Eiland and McEwen, 2012). Hence for this study CCL exposure was chosen to exacerbate MS-induced DA release.

1.10 Serotonin (5-HT) and depression

About 50% of 5-HT cells in the rat brain are found in the DRN which is located in the brainstem (Azmitta and Segel, 1978; Jacobs and Azmitta, 1992). 5-HT exerts its effects through several 5-HT receptors made up of different subtypes that belong to the family of G-protein coupled receptors except 5-HT_3R, which is a ligand-gated ion channel. Examples of 5-HT receptor subtypes include 5-HT_{1A}R, 5-HT_{2A}R, 5-HT_{2C}R, and 5-HT_4R, (Paul and Lowry, 2013). Functionally, 5-HT is involved in arousal (sleep and wake cycle), regulation of mood and emotional behaviours, motor function and appetite (Owen and Nemeroff, 1994; Di Matteo et al., 2008). 5-HT mediates dopamine release both in the PFC and NAc (Di Matteo et al., 2008). Furthermore, interaction of 5-HT and dopamine, in particular in the NAc has been implicated in mechanisms underlying substance abuse and depression (Waselus et al., 2011).

The DRN innervates many brain regions that respond to stressors (Molliver, 1987; Peyron et al., 1998). The 5HT-ergic system is implicated in many mental disorders including anxiety and depression (Owen and Nemeroff, 1994; Blier and de Montigny, 1999; Mann, 1999). Moreover, reduced levels of 5-HT or its precursor (tryptophan) are implicated in the pathophysiology of depression, panic and obsessive-compulsive disorders, OCD (Meltzer and Lowy, 1987; Owen and Nemeroff, 1994).

Different patterns of 5-HT responsivity following various forms of stress are observed. Serotonin levels can increase, decrease or have no response following exposure to different stressors such as restraint stress, forced swimming and motor activity (Kirby et al., 1995; Kirby and Lucki, 1997; Adell et al., 1997). In a validated genetic model of depression, FSL rats showed a decrease in 5-HT turnover in the NAc compared to the Flinders Resistant Line (FRL) rats (Zangen et al., 1997). Also in FSL, it was shown that dopamine release was altered after blocking 5-HT_{2C} receptors (Dremencov et al., 2005). Similarly, changes in the concentration of 5-HT have also been noted in the MS model (Mathew et al., 2001). Increased 5-HT_2R density in the brains of depressed suicide victims have been reported and antidepressant treatment decreases 5-HT_2 receptor density in the brain (Mongeau et al., 1997; Rosel et al., 2000). Moreover, agomelatine, a 5-HT_{2C}R antagonist, induces antidepressant like effects (Loo et al., 2002). Low levels of extracellular DA levels in the NAc of FSL were reversed by local administration of a 5-HT_{2C}R antagonist (Dremencov et al., 2005). Antidepressants may regulate dopamine release through activation of 5-HT receptors (Blier and Bergeron, 1995; Briner and Dodel, 1998; Ichikawa and Meltzer, 1999; Di Matteo et al., 2001; Dremencov et al., 2004).

Pharmacological treatments are aimed at enhancing 5HT-ergic neurotransmission in order to improve depressive moods (Kent et al., 2002; Blier and de Montigny, 1994). This is achieved by blocking 5-HT transporters from taking up 5-HT into the presynaptic terminal and thus resulting in increased concentration of 5-HT in the synaptic cleft (Khwawam et al., 2006; Danese and Pariente, 2008; Paul and Lowry, 2013). This enhances or lengthens exposure of 5-HT to postsynaptic 5-HT receptors thereby increasing 5-HT neurotransmission, which has been shown to be beneficial in treating depression (Di Matteo et al., 2001; Dremencov et al., 2004; Khwawam et al., 2006; Danese and Pariente, 2008).

Furthermore, 7 day treatment with nefazodine (5-HT_{2C}R antagonist), a fast onset antidepressant, normalised immobility time in the FST in FSL rats compared to a classical antidepressant, desipramine (Dremencov et al., 2004). Moreover, nefazodine caused a significant increase in dopamine levels in the NAc in FSL rats that previously had low levels of DA compared to treatment with desipramine

(Dremencov et al., 2004). It has been consistently shown that nefazodine enhances 5-HT by blocking 5-HT_{2C}R levels that are often increased in depression thereby causing a dopamine release in the NAc (Moreo et al., 1996; Sanchez and Hyttel, 1999; Dremencov et al., 2004). Therefore enhanced 5-HT levels either by normalising or a down-regulating 5-HT_{2C}R level improves depressive-like behaviours (Moreo et al., 1996; Dremencov et al., 2004).

These studies provide evidence that the 5HT-ergic system is involved in depression. For this study the hypothalamus and PFC were chosen to investigate 5-HT levels after MS and CCL due to the fact that the both PFC and hypothalamus form part of the HPA axis stress system and are most vulnerable to the effects of early life and adult stress exposure (Lupien et al., 2000; Geoffroy et al., 2006; Albers et al., 2008; Lupien et al., 2009). Furthermore, the SCN which is part of the hypothalamus plays a crucial role in controlling stress responses; more specifically in regulating glucocorticoid release and their circadian rhythms (Campbell and Dawson, 1990; Redlin, 2001; Liu et al., 2006; Bao et al., 2008). Most importantly, 5-HT also interacts with other brain regions implicated in depression, such as the NAc, further supporting the importance of studying 5-HT levels in depression after MS and CCL exposure (Ichikawa and Meltzer, 2000; Di Matteo et al., 2001; Dremencov et al., 2004).

1.11 Opioid and depression

The opioid peptide system is made up of endorphins, enkephalins, and dynorphins that act on three receptor subtypes, namely MOR, delta-opioid (DOR) and kappa-opioid (KOR). Opioid peptides and receptors are widely distributed in several brain regions in the brain, including the limbic system (amygdala, NAc, and hypothalamus) and pituitary glands to regulate many biological processes of which include nociception, analgesia and feeding (Akil et al., 1998; Terenius, 2000; Przewlocki, 2002). The opioid system regulates many biological systems that respond to stress and these include behavioural, endocrine and autonomic nervous system (Calcagnetti and Holtzman, 1992; Watkins et al., 1992; Vanderschuren et al., 1995; Drolet et al., 2001).

The role of this system in controlling feeding is believed to be mediated through the NAc, an important brain area that is crucial for regulating motivation, reward process and pleasure seeking behaviours (Koob and Bloom, 1988; Ikemoto and Panksepp, 1999; Shirayama and Chaki, 2006). Injection of MOR and DOR agonists into the NAc and VTA evokes feeding (Kelley et al., 2002; Baldo and Kelley, 2007). In particular, MOR agonists function in the NAc is dependent on both the taste and the macronutrient of food; especially high energy food (Kelley et al., 2002; Baldo and Kelley, 2007). To lend support to MOR in regulating feeding, it was reported that MOR KO mice displayed decreased motivation to eat (Papaeo et al., 2007).The opioid system is also involved in the rewarding effects induced by drugs of abuse and sexual behaviours. For example, MOR KO mice were unresponsive to the effects of morphine and heroin during conditioned place preference (CPP) and self-administration (Matthes et al., 1996; Becker et al., 2000; Marquez et al., 2007). Moreover, injection of a KOR antagonist into the NAc increased female directed behaviour to male rats (Matuszewich et al., 1995). These findings indicate that the NAc is an important brain region mediating opioid functions.

The NAc is also implicated in the pathophysiology of depression (Ikemoto and Panksepp, 1999). Furthermore, it is often observed that depressed individuals show appetite and weight disturbance, reduced motivation and energy levels suggesting that the opioid system might be involved in aetiology of depression (Ikemoto and Panksepp, 1999; Pecina and Berridge, 2000; American Psychiatric Association, 2005). Individuals who committed suicide display enhanced MOR levels (Scarr et al., 2012). Furthermore, children whose mothers were taking opiates during pregnancy were reported to exhibit elevated anxiety and aggressiveness, providing evidence of the role of the opioid system in mood disorders (de Cubas and Field, 1993).

Opioid receptors also regulate activity of the HPA axis, the KORs and DORs are highly expressed in the hypothalamic nuclei and decreased HPA axis activity was reported in MOR KO mice that were subjected to chronic restraint stress (Wang et al., 2002). MOR and KOR have been reported to mediate cardiovascular responses during various forms of stress (Marson et al., 1989). For example, pre-

treatment with a MOR-antagonist prevents increases in blood pressure caused by stress exposure (Jimenez et al., 1990; Kapusta et al., 1989). Similarly, DOR activation by several agonists in rats and mice induces antidepressant-like effects in various behavioural tests of such as the FST and learned helplessness test (Tejedor-Real et al., 1995; Naidu et al., 2007). Administration of an opioid agonist induces preference for the open arms and increases exploration activity during the EPM test, indicative of anxiolytic behaviours (Broom et al., 2002; Terregrossa et al., 2006). DOR KO mice show increased anxiety levels and depressive-like behaviour whereas DOR agonists decreases immobility time in the FST (Broom et al., 2002; Terregrossa et al., 2006). Moreover, KOR antagonists decrease immobility time in the FST whereas KOR agonists increase immobility time in the FST (Mague et al., 2003).

The opioid system has also been implicated in maternal care and it has been found that MOR function in maternal care is important for maintaining stress responses and stress-related disorders throughout life (Liu et al., 1997). Variants within the MOR gene are associated with the quality of parental attachment in primates and humans (Barr et al., 2008; Copeland et al., 2011). For example, increased ultra sound vocalizations as a result of MS are reduced in MOR KO mice, suggesting that the MOR activity is implicated in mood and stress regulation (Moles et al., 2004). Similarly, MOR KO mice are less anxious; exhibit reduced depressive-like trait features than wild-type mice (Filliol et al., 2000; Yoo et al., 2004). In contrast, reduced MOR levels in the NAc were reported after MS which were restored by CCL exposure, suggesting that MS induces depressive like behaviours (Dimatelis et al., 2012).

It is evident that the opioid system is involved in the pathophysiology of depression. However, the contradictory findings on the function of MOR in depression suggest that there is little known about the exact function of this receptor in the pathophysiology of depression (Filliol et al., 2000; Yoo et al., 2004; Moles et al., 2004; Dimatelis et al., 2012). Hence, MOR protein levels in the NAc were chosen to investigate the aetiology of depression in rats subjected to MS and exposed to additional stressor (CCL). Of importance to note is the prominent role of the MOR activity in mediating feeding, motivation, and mood functions within the

NAc (Liu et al., 1997; Shirayama and Chaki, 2006; Baldo and Kelley, 2007; Dimatelis et al., 2012). Further lending support for choosing the NAc as the brain region of interest for investigating MOR function in depression.

1.12 Orexin and depression

The orexinergic system is made up of approximately 50 000 - 80 000 orexin producing neurons in the human brain (Thannickal et al., 2000; Fronczek et al., 2005; Peyron et al., 1998). The orexinergic system controls many functions, including feeding, reward process and HPA axis activity (de Lecea et al., 1998; Cason et al., 2010; Aston-Jones et al., 2010). For example, orexin neurons in the VTA, amygdala and NAc are believed to regulate stress, reward and motivation and in the LH, orexin neurons regulate reward seeking behaviours (Fadel et al., 2002; Harris and Aston-Jones, 2006). The orexinergic system includes orexin A and B peptides and orexin receptor 1 and 2 (OXR-1 and OXR-2) which are found in many parts of the brain (Sakurai et al., 1998; de Lecea et al., 1998; Lee et al., 1999; Thannickal et al., 2000; Fronczek et al., 2005). Orexin A and B are hypothalamic neuropeptides found on the lateral hypothalamus (LH) where they are involved in regulating sleep, arousal and feeding (de Lecea et al., 1998; Sakurai et al., 1998; Sakurai et al., 2010; Cao and Guilleminault, 2011).

Orexin peptides bind specifically OXR-1 and OXR-2 receptors also known as hypocretin 1 and hypocretin 2 respectively (Sakurai et al., 1998). OXR1 binds very strongly to orexin A compared to orexin B (Sakurai et al., 1998). On the other hand OXR-2 binds both peptides with high affinity. OXR-1 mRNA is expressed in many brain regions such as the hippocampus and hypothalamus (e.g. PVN and ventromedial hypothalamus). OXR-2 mRNA is also expressed in many brain regions such as the cerebral cortex, hippocampus, DRN and many hypothalamic regions (Marcus et al., 2001; Sakurai et al., 1998). All of these areas have been implicated in the mediation of depressive-like behaviours.

The orexinergic system is involved in several processes that are dysregulated in depressive subjects including the sleep/wake cycle (Sakurai et al., 2010; Cao and

Guilleminauit, 2011). The sleep/wake cycle is regulated by the orexinergic system that receives input from the retina (Hattor et al., 2003). For example, abnormal circadian rhythms in orexin levels were found in the cerebrospinal fluid (CFS) in patients with SCN lesions (Deboer et al., 2003). Despite the fact that orexin neurons do not receive direct input from the SCN, it is believed that the orexin neurons are extensively innervated by input coming from the bed nucleus of the stria terminalis (BNST), PVN and dorsomedial hypothalamus brain areas that receive direct input from the SCN (Sakurai et al., 2005; Yoshida et al., 2006).

Sleep disturbances and circadian abnormalities are some of the symptoms reported in depressed patients and orexinergic system as an important regulator of the sleep and wake cycle has been implicated in dysregulated sleep/wake cycle during depression (Peyron et al., 2000; Salomon et al., 2003; Sakurai et al., 2010; Cao and Guilleminauit, 2011). Similarly, orexin deficiency causes sleep disorders and narcolepsy in both humans and animals which is indicative of the important role of this system in maintaining the sleep/wake cycle (Peyron et al., 2000; Thannickal et al., 2000; Salomon et al., 2003; Liu et al., 2011). Narcoleptic individuals show a significant decrease in the number of orexin neurons (90%) as well as displaying decreased levels of orexin A in cerebrospinal fluid (Peyron et al., 2000; Mignot et al., 2002; Salomon et al., 2003; Liu et al., 2011). Furthermore, polymorphisms within OXR-1 gene were associated with increased risk for developing unipolar disorder (Rainero et al., 2011). Individuals with anxiety accompanied by panic attacks have increased levels of orexin in the CSF (Johnson et al., 2010).

In response to stress, orexin neurons receive input from the limbic system and are also various forms of stress such as foot shock, food deprivation and social defeat stress (Sakurai et al., 1998; Ida et al., 2000; Winsky-Sommerer et al., 2004; Sakurai et al., 2005; Yoshida et al., 2006 Berridge et al., 2010). During stress, orexin neurons also regulate HPA axis activity by innervating CRH neurons in the PVN of the hypothalamus (Winsky-Sommerer et al., 2004). It is believed that the relationship between the CRF system and the orexinergic system might be useful in maintaining wakefulness during stressful events (Winsky-Sommerer et al., 2004).

Mice subjected to unpredictable chronic mild stressors (UCMS) displayed decreased levels of cell proliferation and neurogenesis in the hippocampus which was reversed by chronic fluoxetine treatment and dual orexin receptor (OXR-1 and OXR-2) antagonist (Nollet et al., 2012). Furthermore, both fluoxetine and dual orexin (OXR-1 and OXR-2) antagonist administration improved depressive-like behaviours, suggesting that inhibition of the orexinergic system has antidepressant-like effects (Nollet et al., 2012).

Nocjar et al. (2012) reported reduced levels of orexin in the VT, mPFC and hypothalamus of rats subjected to a social defeat model. Furthermore, these rats displayed a decreased sucrose preference scores when matched to controls, which was attributed to the decreased levels of orexin in the dopamine reward system (Nocjar et al. (2012). In agreement with these findings, decreased orexin function was also associated with diminished reward seeking behaviour, indicating anhedonic behaviour (Aston-Jones et al., 2002). Moreover, a genetic animal model of depression, Wistar-Kyoto rats, exhibited an 18 % decrease in the number of orexin neurons compared to controls (Allard et al., 2004). In contrast, FSL rats had high number of orexin neurons in the hypothalamus including increased immobility in the FST (Mikrouli et al., 2011). The discrepancies in these studies might due to the fact that Mikrouli et al. (2011) used female rats in contrast to the male rats that were used by Allard et al. (2004). Furthermore, the female rats were subjected to forced swimming (stressor) before the stereological analysis of orexin neurons which is most likely to have contributed the increased number of orexin neurons reported in this study (Mikrouli et al., 2011).

These findings provide substantial evidence of the role of the orexinergic system in depression. It is well established that depressed individuals exhibit many depressed moods (altered sleep, changes appetite, and anhedonia) that are also regulated by this system, indicating that the orexinergic system is implicated in the pathophysiology of depression. Despite the improvement in understanding the function of the orexinergic system in depression, the contradictory findings reported on the role of orexin peptides and receptors suggest that more research is required to provide more insight on the function of this system in depression (Allard et al., 2004; Mikrouli et al., 2011; Nocjar et al., 2012). Hence, for this study

orexin peptides (A and B) and orexin receptors (OXR-1 and OXR-2) were chosen, to investigate the role of the orexinergic system in rats subjected to MS and additional CCL stress paradigm.

1.13 Hypothesis

Exposure to CCL for 3 weeks will result in changes in glutamate- and potassium-stimulated [^{3}H]dopamine release, 5-HT levels, MOR-1 levels, orexin (A and B) and OXR-1 and 0XR-2 levels in the brains of MS rats (Figure 1.5).

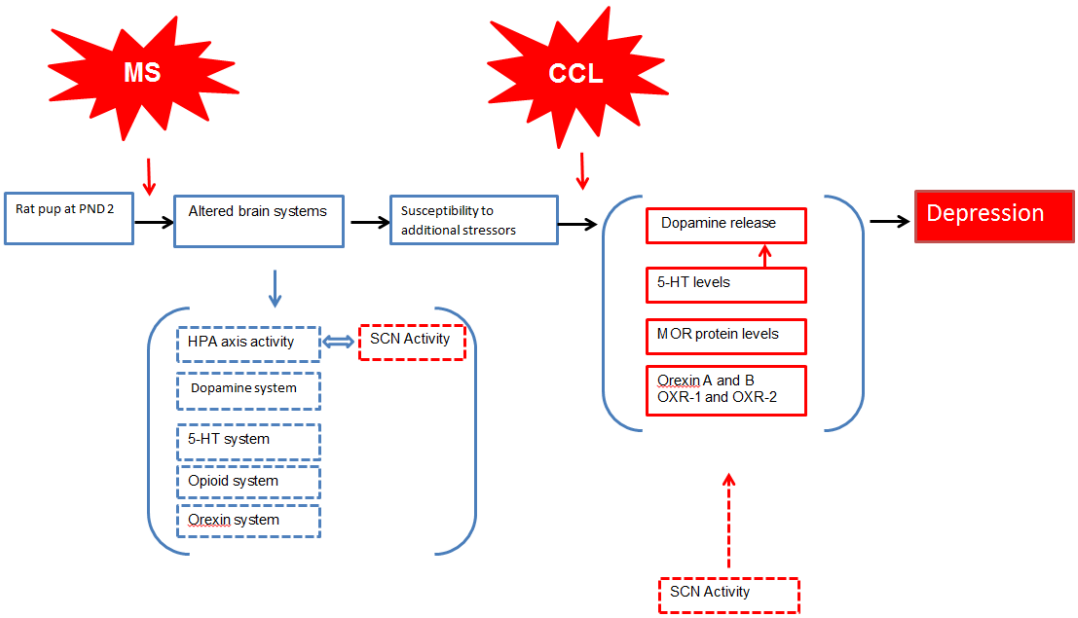


Figure 1.5: Hypothesis. It is hypothesised that MS exposure (for 2 weeks) starting from postnatal day (PND) 2 during a critical stage (i.e. postnatal period) in brain development will alter the HPA axis activity. As the hippocampus, frontal cortex and amygdala; important brain areas making up the stress system are still maturing during this period, suggesting that the HPA axis is vulnerable to the effects of stress exposure. Moreover, an altered HPA axis might impair SCN activity as these systems influence each other in responding to stress effects. Furthermore, MS exposure is most likely to impair dopamine system, serotonin (5-HT), opioid system and orexin system, brain systems that are implicated in stress regulation. Alterations to these systems predispose the brain to additional stressors later in life. As a result, CCL exposure is likely to exacerbate the effects of MS thereby changing dopamine release in the NAc, 5-HT levels in the hypothalamus and prefrontal cortex (PFC) and μ-opioid receptor (MOR) levels in the NAc. Orexin peptides (A and B) in the hypothalamus and orexin receptors in the PFC are most likely to be affected by additional exposure to CCL. Furthermore, SCN activity is also vulnerable to the effects of additional CCL exposure suggesting that biological functions that are dependent on SCN activity might also be altered by CCL exposure thereby triggering a depressive episode.

1.14 Aims and objectives

This study aims to explore the effects of additional CCL exposure in an MS model by:

- Measuring glutamate- and potassium-stimulated [^{3}H]DA release in the NAc in the four experimental groups; non-maternally separated (NMS) rats, NMS rats subjected to CCL, MS rats and MS rats exposed to CLL.

- Testing the effects of CCL on 5-HT levels in the hypothalamus and PFC

- Measuring MOR-1 levels in the NAc

- Measuring orexin peptides A and B and OXR-1 and OXR-2 levels in the PFC.

CHAPTER 2: METHODOLOGY

2.1 Animals

A total of 112 male Sprague Dawley (SD) (Rattus Norvegicus) comprising of 28 rats per group; NMS rats, NMS rats exposed to 3 weeks of CCL, MS rats and MS rats subjected to CCL were used for this study. Half of each experimental group (n=14/group) was used for the in vitro superfusion experiments and the rest (n=14/group) was used for biochemical analysis (Figure 2.1). All experimental procedures conducted were approved by the Health Sciences Faculty Research Ethics Committee of the University of Cape Town (Ethics reference number 021/057) (Appendix A). The date of birth was regarded as postnatal day zero (PND 0). All the animals were kept under standard conditions which included a constant room temperature (23 ± 1 °C), tap water and rat chow was available ad libitum for the duration of the experiment. A 12 h/12 h L/D cycle was maintained (lights on from 06:00 and off at 18:00) except during the CCL procedure where a 12 h/12 h L/L cycle was maintained.

Rats were housed not more than four in Plexiglas cages with sawdust as bedding. All rats were housed in the same colony room separate from the experimental rooms in which the MS and CCL occurred.

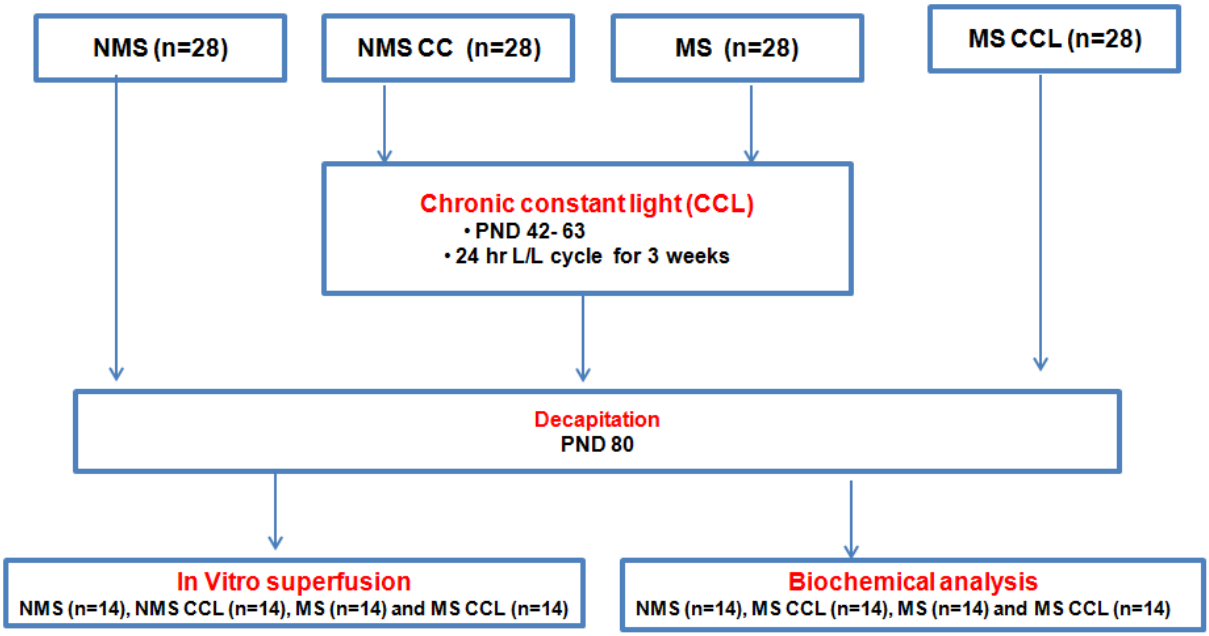


Figure 2.1: Overview of experimental procedures. Four experimental groups out of which two groups; non-maternally separated (NMS, n=28) rats and maternally separated (MS, n=28) rats were exposed to CCL (24 hr L/L cycle) for 3 weeks (from postnatal day, PND, 42-63) during adolescence and the remaining two groups; NMS (n=28) rats and MS (n=28) animals were not subjected to CCL. At PND 80, all the rats were decapitated and half of the rat brains (n=14) from each experimental group were used to dissect out the designated brain areas employed for in vitro superfusion and biochemical analysis.

2.2 Maternal Separation (MS) model

On PND 2, pups were sexed and culled to 8 rats per litter (Figure 2.2). Male rats were used in the study as it has been previously demonstrated that MS affects the HPA axis activity of male rats and thus increases the likelihood of altering the HPA axis activity (Hout et al., 2001; Ladd et al., 2004; Veenema et al., 2006). Equal litter sizes ensured that pups get adequate nutrition from their mothers. The MS paradigm was performed according to the procedure established by Ladd et al. (2000) with slight modifications. The mother was removed from the litter and placed in a different cage whilst the pups remained in the home cage. The cage of the mother remained in the housing satellite facility and the home cage containing pups was taken to a different room. MS was performed for 3 hours daily, from 09h00 – 12h00 during the first 2 weeks of life (PND 2 to 14) (Figure 2.2). During

MS, pups were kept under infrared light with temperatures kept between 30 - 33 °C in order to prevent hypothermia. After 3 hours of separation, the pups were returned to the animal housing satellite facility and the dam was returned to the home cage with the pups and reunited with her pups. From PND 15, pups were reared under normal conditions (Figure 2.2).

The cages were cleaned twice a week and care was taken to ensure that pups were not handled as handling reverses MS-induced changes (Levine, 1957; Fernandez-Teruel et al., 2002; Kiosterakis et al., 2009). Neonatal handling has been shown to induce a resilient HPA axis, thus increasing the ability to cope and adapt to stressful events (Levine, 1957; Fernandez-Teruel et al., 2002; Kiosterakis et al., 2009).

NMS rats were not separated from their mothers instead they were reared under standard conditions.

2.3 CCL exposure

Two groups of male SD rats, half of the NMS and MS rats were subjected to CCL for 3 weeks (PND 42 to PND 63). The CCL exposure was performed in a dark room where the home cage was placed under lights with light intensity ranging between 100-120 lux generated by a 40W bulb. After the CCL exposure rats were returned to the animal housing facility with standard lighting conditions; 12 h/12 h light/dark cycle (lights on at 06:00 and off at 18:00). At PND 80 all the animals were decapitated (Figure 2.2).

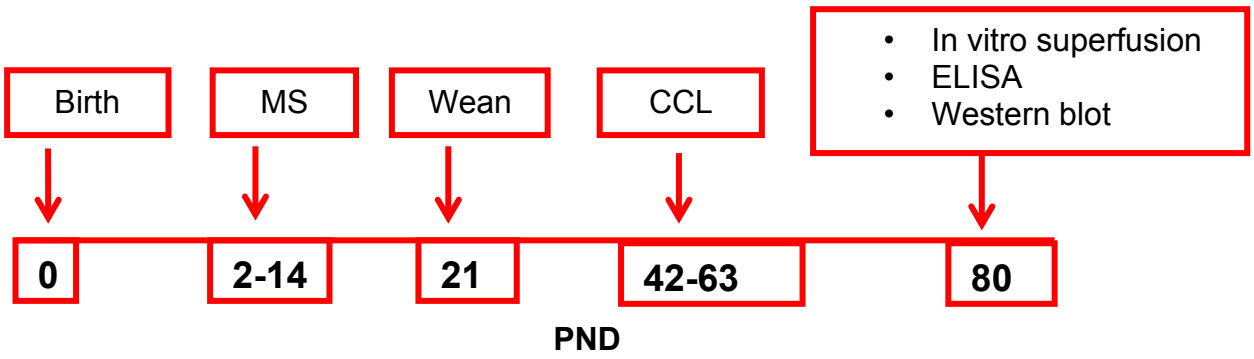


Figure 2.2: Timeline of experimental procedures. Timeline of the experimental procedures conducted from postnatal day (PND) 0 to 80. **Abbreviations**: Maternal separation (MS), CCL (chronic constant light) and enzyme-linked immunosorbent assay (ELISA).

2.4 Accumbal [^{3}H]DA release measurement by in vitro superfusion technique

> ❖ See the Appendix section for the list of buffers, reagents and additional materials used in this protocol.

An in vitro superfusion technique that has been standardised in our laboratory prior to the present study was employed to measure glutamate- and potassium-stimulated [^{3}H]DA release from NAc slices (Russell and Wiggins, 2000; Howells and Russell, 2008; Sterley et al., 2012) (Figure 2.3). On PND 80 rats were killed by decapitation between 09h00-12h00. After decapitation the brain was rapidly removed and placed in ice-cold Krebs buffer for 20 minutes. The chilled brains were sliced coronally with a McIlwain tissue chopper (Figure 2.4). The NAc shell and core were dissected from the two 0.9 mm^2 brain slices that contained the NAc. The tissue was chopped into 0.3 X 0.3 mm sections and suspended in Krebs

buffer for 10 minutes at 37 °C (Appendix B1). The Krebs buffer was aerated with carbogen (95 % O_2 / 5 % CO_2) for the duration of the experiment.

The NAc core and shell tissue slices were then incubated for 15 minutes with 0.125 µM [^{3}H]DA (2.67 µl – [7-3H], 9.25 MBq/ ml, 21.2 Ci/mmol, Amersham biosciences UK limited) in the presence of 5.67 mM ascorbic acid to reduce the metabolism of [^{3}H]DA. This ensures that the labelled [^{3}H]DA is taken up into the dopaminergic terminals in the NAc slices. Immediately after the incubation period, equal portions of the tissue were transferred to superfusion chambers and continuously perfused with Krebs buffer at 37 °C. Each experiment consisted of 2, 3 or 4 rats. The tissue from each rat was distributed over four superfusion chambers (Figure 2.5). Two out of the 4 chambers per rat were loaded with tissue from the NAc shell and the other 2 chambers were loaded with tissue from the NAc core. This was carried out so that there was a duplicate determination of [^{3}H]DA release from each subdivision of the NAc from each rat. DA release was induced either by exposure to 1 mM glutamate or 25 mM potassium stimulation at specific time points during the experiment (Figure 2.5).

The superfusion experiment was composed of the following components; two 45-minute washes to remove excess radioactive material and to achieve a stable efflux of radioactivity (Figure 2.5). This was followed by two 5-minute fractions of eluate to establish baseline release of [^{3}H]DA. The third 5-minute was performed by removing the inlet tubes from the Krebs buffer into the 1 mM glutamate containing Krebs buffer for glutamate stimulation. Glutamate stimulation was achieved by exposing the tissue slices to 1 minute pulse of the 1 mM glutamate to stimulate [^{3}H]DA release from the NAc shell and core (Figure 2.5). After the 1 minute glutamate stimulation, the inlets tubes were returned to the Krebs buffer for the remaining 4 minutes. An additional three 5-minute fractions of eluate were collected to re-establish the baseline of [^{3}H]DA release after glutamate stimulation (Figure 2.5). This was followed by a 25 mM potassium stimulation which was achieved by removing the inlet tubes from the Krebs buffer to potassium containing Krebs solution for 1 minute. For the remaining 4 minutes, the inlet tubes were returned back to the Krebs buffer to collect a 5-minute fraction of [^{3}H]DA release (Figure 2.5). A further two 5-minute fractions of eluate were collected to

re-establish a baseline of [^{3}H]DA release following the potassium stimulation. At the end of these two 5-minute fractions the superfusion chambers were allowed to drip dry before collecting the pellet (cotton wool containing radio-labelled tissue slices) (Figure 2.5).

The pellet was collected into a vial containing 1 ml of 0.1 M of NaOH which was added in order to break the tissue slices thus releasing the remaining endogenous [^{3}H]DA. The eluate and pellets were left overnight and counted with a beta counter the next day.

Before samples were counted, 3.4 ml of scintillation fluid was added to each vial in order to measure the radioactivity labelled DA in the samples. The function of the scintillation fluid is to transfer the energy of the beta particles that are emitted by the radioactive isotope [^{3}H] of samples to the scintillation analyser (Kessler, 1991). After the addition of the scintillation fluid, radioactivity in the 5-minute fractions of eluate and radioactivity remaining in the tissue slices at the end of the experiment were determined using a Packard 1900 TRI-CARB liquid scintillation analyser. The measured radioactivity (disintegrations per minute, DPM, 10 minutes per sample) in each vial was determined using the quench curve generated from the known radioactivity of the quenched standard samples (Appendix B2). Briefly, the quench curve was generated by plotting the quench values of the standards, represented by the transformed external standard spectrum (tSIE) against the percentage efficiently. The percentage efficiency refers to how efficient the energy of the beta particles is transferred to the scintillation analyser (Kessler, 1991). From this, the DPM of the radio-labelled samples was quantified. The DPM values of the samples where within the quench curve of the standards, between 200-400 tSIE values (Appendix B2).

To correct for the variability in the amount of tissue in the superfusion chambers, radioactivity in each eluate fraction was expressed as a percentage of the total amount of radioactivity present in the slices at the time of release of that 5 minute fraction. To determine the glutamate- or potassium-stimulated [^{3}H]DA release relative to the baseline, the percentage release in the preceding baseline fraction was subtracted from the percentage release in the stimulation fraction (Figure 2.6).

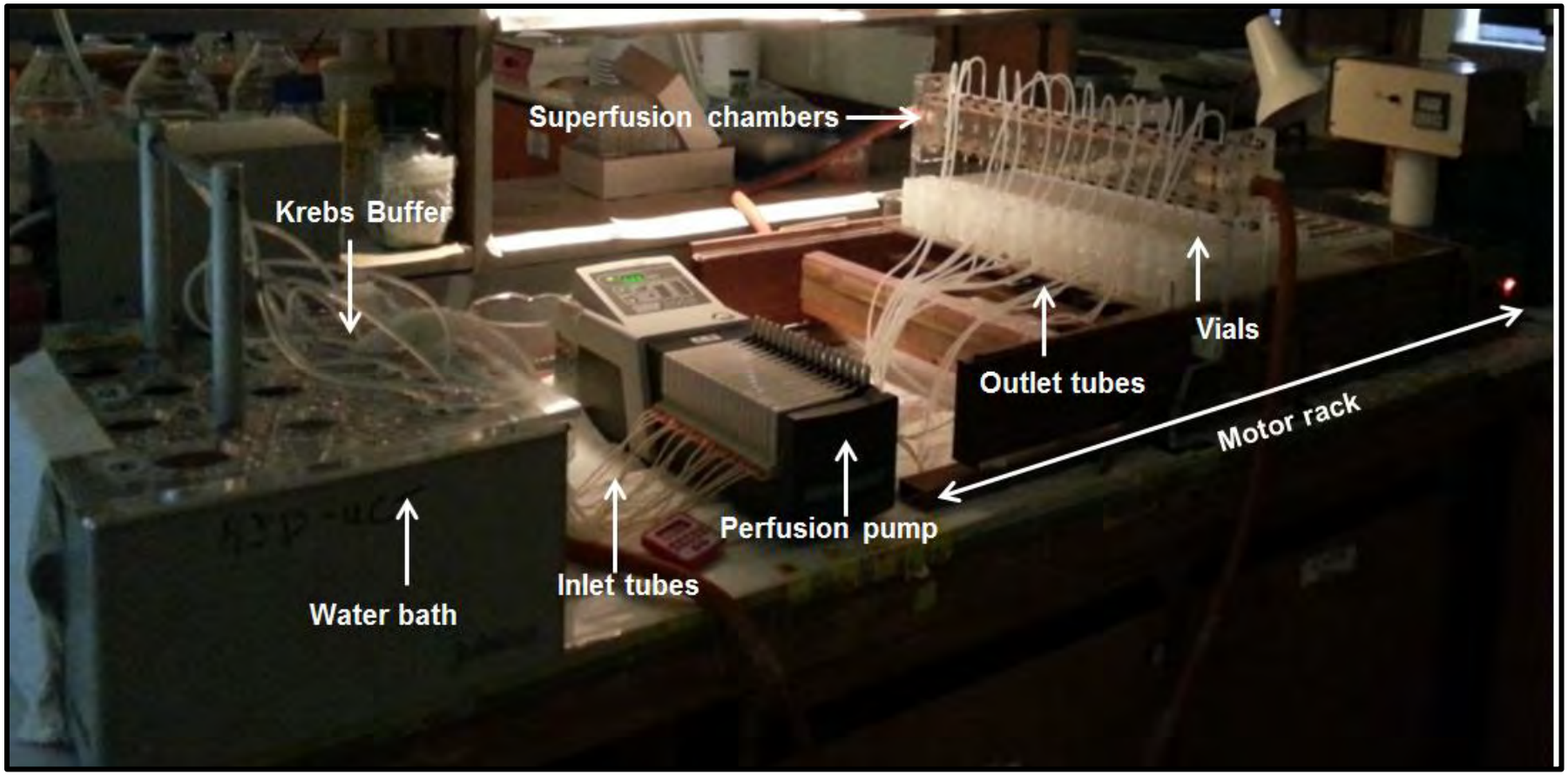
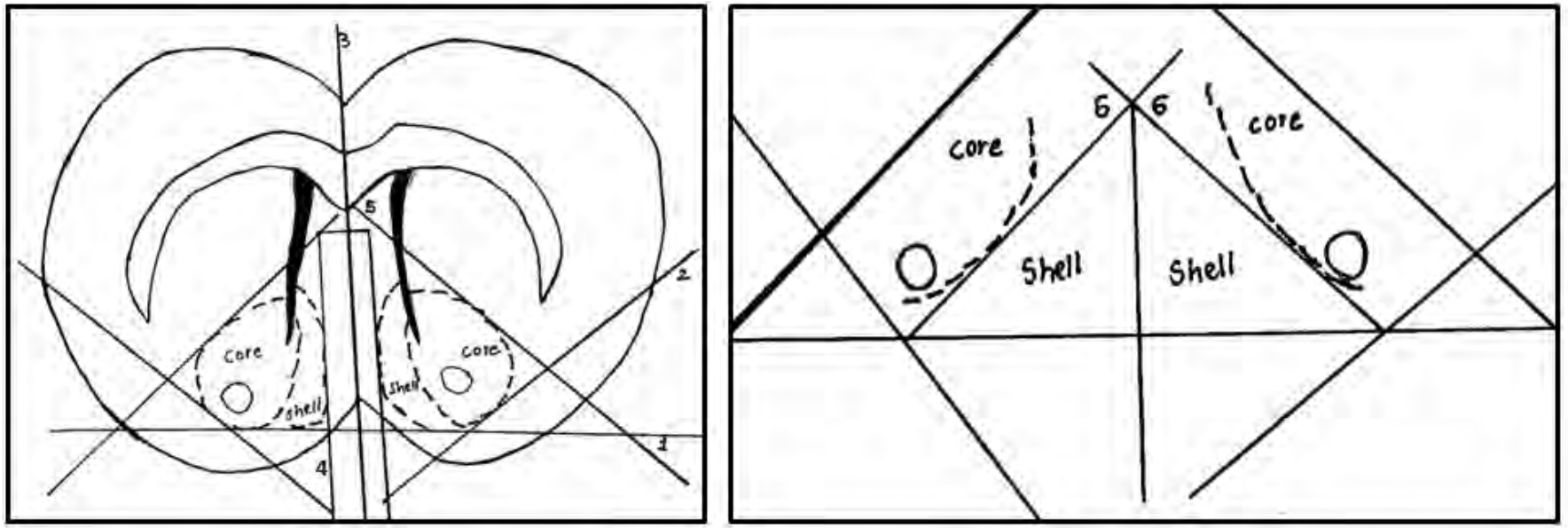


Figure 2.3: In Vitro superfusion setup. The setup of the in vitro superfusion technique used to measure glutamate- and potassium-stimulated [^{3}H]DA release in the NAC core and shell.



(Adapted from paxinos and Watson, 1986)

Figure 2.4: A dissection diagram of the NAc core and shell. Following decapitation, 0.9 mm^2 coronal slices of the brain were obtained using a McIlwain tissue chopper. The two slices containing the NAc were used to dissect the NAc shell and core. The remaining olfactory bulb and surrounding tissue was removed with a blade as indicated by cut number 1. The corpus callosum, NAc (core and shell) and anterior commissures were used as markers to determine cut number 2, the slice was then cut in half (3) in order to make a precise dissection of the NAc core and shell. The brain tissue medial to the NAc was removed (cut number 4). Cut numbers 5 and 6 indicate how the NAc core and shell were selectively dissected.

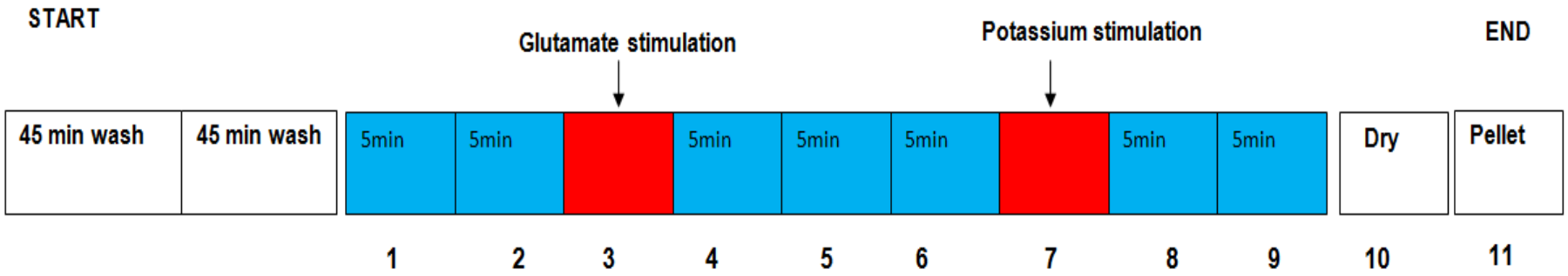


Figure 2.5: A schematic diagram showing the in vitro superfusion experiment set up. Two 45-minute washes were established to remove excess radioactive material. Number 1 and 2 are two 5-minute fractions of [^{3}H]DA release for baseline recordings. Number 3 represents 5-minute fraction of [^{3}H]DA release divided into 1-minute pulse of 1 mM glutamate-stimulation and remaining 4 minutes of [^{3}H]DA release in Krebs buffer solution. Number 4 to 6 represent 5-minute fractions of [^{3}H]DA release collected to re-establish baseline following the 1mM glutamate stimulation. Number 7 represents 5-minute fraction of [^{3}H]DA release consisting of 1-minute 25 mM potassium stimulation and the remaining 4 minutes of Krebs buffer solution. The last two of 5- minute fractions (numbers 8 and 9) were collected to re-establish baseline [^{3}H]DA release following the 25 mM potassium stimulation. Number 10 represents a time point in which the perfusion pump was switched off to allow the superfusion to drip dry before the pellet was collected. The collection of the pellet to measure the total radioactive DA left in the endogenous DA stored is represented by number 11.

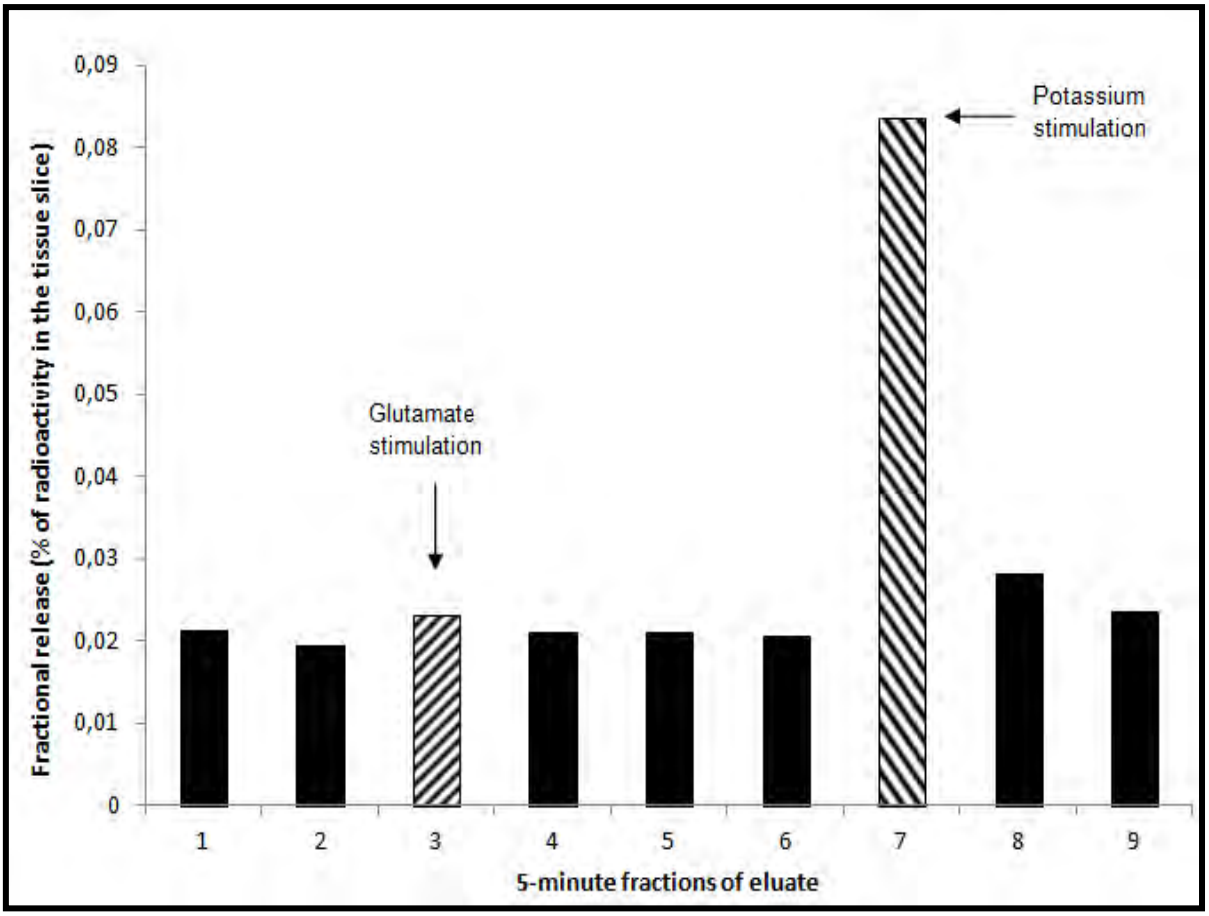


Figure 2.6: An example of the 5-minute fractions of eluate collected during the in vitro superfusion experiment. Number 3 on the x-axis contained glutamate-stimulated release of radioactivity and number 7 contained potassium-stimulated release of radioactivity. The remaining 5- minute fractions are baseline recordings of [^{3}H] DA release from the NAc shell and core pre and post stimulated [^{3}H]DA release.

2.5 Biochemical Analysis

2.5.1 Measurement of 5-HT levels in the hypothalamus and PFC using an ELISA assay

> ❖ See the Appendix section for the list of buffers, reagents and additional materials used in this protocol.

On PND 80 rats were killed by decapitation between 09h00-12h00. After decapitation brain tissue was rapidly removed and placed in cold saline (0.9 % NaCl) solution. The whole hypothalamus was dissected manually using surgical instruments and this was followed by a dissection of the PFC. PFC was dissected from coronal slices (0.9 mm^2) obtained with a McIlwain tissue chopper. The tissue samples were stored in liquid nitrogen until analysed.

Hypothalamus and PFC tissue was weighed and diluted with RIPA buffer (Appendix C1). Protease inhibitor cocktail (2 µL per 1 ml of RIPA buffer, Thermo Scientific, Rockford, USA) was added to inhibit protein degradation. Furthermore, 1 mM EGTA was added to the buffer solution to assist tissue extraction by disrupting protein complexes that contain proteins of interest (Tria et al., 2013). RIPA buffer (1 ml) was added per 50 mg of hypothalamus and PFC sample. Proteins were then solubilised by sonicating the samples for 10 seconds and mixing them on a vortex for another 10 seconds. After which, they were then left to stand on ice for 20 minutes. After 20 minutes, the samples were centrifuged at 17200 x g for 30 minutes at 4 °C and the supernatant was collected and kept overnight at -80 °C.

The next day, all the samples were centrifuged again at 12000 x g for 10 minutes at 4 °C. Twenty µl from the samples and from each of the two controls (1 and 2) were added to a glass tube. A further 100 µl of Assay buffer and 25 µl of acylation reagent (Appendix C2) were added to the glass tube and mixed using a vortex

mixer. The glass tubes were then covered and incubated in the water bath for 15 minutes at 37 °C. Following the incubation period, 2 ml of the Assay buffer was added to each glass tube and mixed on a vortex mixer. The samples were then centrifuged for 10 minutes at 1500 x g. Immediately after centrifugation, 50 µl of each standard (which were already acetylated), control and sample was added to the wells of the microtiter plate which was coated with a primary antibody, anti-rabbit antiserum produced in goat (Appendix C2). This was followed by addition of 50 µl of biotinylated 5-HT and a further 50 µl of rabbit secondary antibody was also added into the microtiter plate for the production of the antibody-antigen complex. This procedure induces a competitive reaction whereby there is a competition between biotinylated 5-HT and the non-biotinylated samples for the fixed number of the goat anti-rabbit (GAR) primary antibody as described in the 5-HT ELISA kit by the manufacturer. The amount of biotinylated antigen bound to the antibody is inversely proportional to the analyte concentration of the sample, thus the more biotinylated 5-HT bound to the antibody, the less concentrated the sample. The microtiter plate was then covered and incubated for 90 minutes at room temperature (18 - 25 °C) using an orbital shaker (500 rotations per minute, (rpm)). After 90 minutes, the plate was washed three times with 250 µl wash buffer to remove non-specifically bound proteins. After which, 150 µl of freshly prepared enzyme conjugate (streptavidin alkaline phosphate) was added to each well. The plate was covered and incubated on an orbital shaker (500 rpm) for 60 minutes at room temperature (18 – 25 °C) to ensure that the enzyme binds to the secondary antibody before adding enzymatic substrate. This was followed by another wash step (3 x 250 µl) with the wash buffer. A 200 µl aliquot of p-nitrophenyl phosphate (PNPP) substrate solution was added into each well and incubated for 60 minutes at room temperature using an orbital shaker at 500 rpm. This causes a catalytic reaction whereby the streptavidin alkaline phosphate acts on the PNPP substrate to produce a colour change, corresponding to the protein concentration. The substrate reaction was stopped by adding 50 µl of PNPP stop solution (1 M NaOH) and shaking the plate to mix the contents in the plate (Appendix C2). After which, the absorbance (optical density, OD) levels were read at 405 nm with a spectrophotometer (Appendix E).

The 5-HT concentration of the controls and samples was read from the standard curve. The 5-HT concentration of the hypothalamic samples was within the standard range as specified in the quality control certificate of the kit (0.08 - 19.8 ng/ml) (Figure 2.7) (Appendix C3). In contrast, the 5-HT concentration of PFC tissue was not within the standard range (0.08 – 19.8 ng/ml) (Figure 2.8) (Appendix C4). Specifically, 2 samples (0.058 and 0.022 ng/ml) from the NMS CCL group and 3 samples (0.026, 0.057 and 0.033 ng/ml) from the MS CCL were below the lowest standard (0.08 ng/ml) (Figure 2.8). A possible explanation for low 5-HT concentrations could be that exposure to CCL in both groups (NMS and MS) might have reduced the 5-HT concentration below that of the lowest standard. Another more plausible explanation for the low values could be as a result of dilution factor as the samples were highly diluted (50 mg wet weight: 1 ml RIPA) with sample preparation before further dilutions (x107) of the samples during the ELISA procedures.

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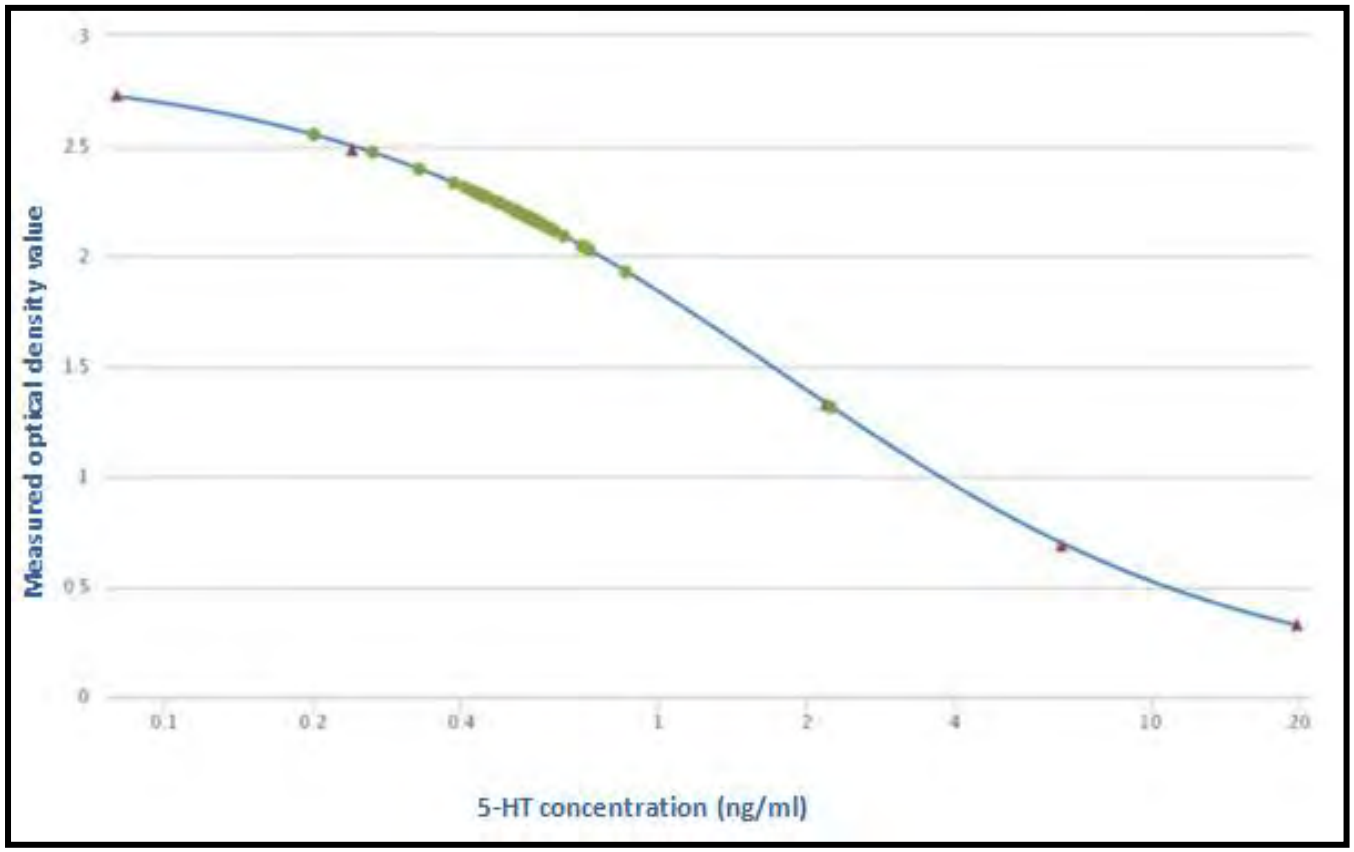
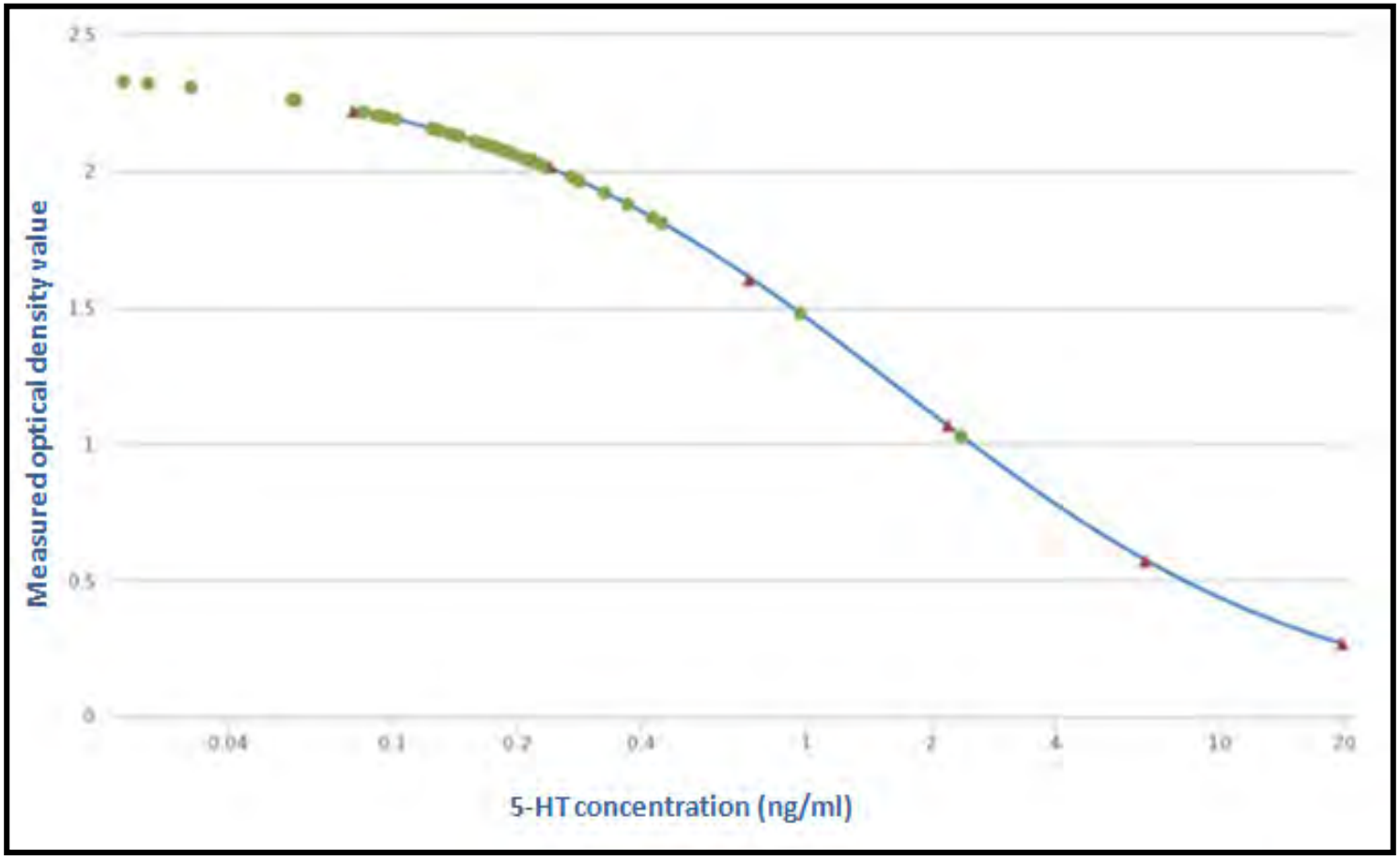


(Standard curve generated using the free online curve *fitting* tool for ELISA analysis, readerfit.com)

Figure 2.7: The standard curve diagram for the 5-HT concentration (ng/ml) in the hypothalamic tissue samples. The 5-HT concentration of the hypothalamic tissue samples (indicated by green data points) is within the concentration range of the standards (0.08-19.8 ng/ml).



(Standard curve generated using the free online curve *fitting* tool for ELISA analysis, readerfit.com)

Figure 2.8: The standard curve for the 5-HT concentration (ng/ml) in the prefrontal cortex (PFC) tissue samples. The 5-HT concentration of the tissue samples of the PFC (indicated by green data points) was not within the range of the standards; as 5 samples, 2 from NMS CCL group and 3 from the MS CCL groups were below the lowest standard (0.08 ng/ml).

2.5.2 Western blotting

On PND 80 rats were killed by decapitation between 09h00 - 12h00. Following decapitation, the whole hypothalamus was dissected and stored in liquid nitrogen until analysed. Coronal slices (0.9 mm^2) of the brain were cut with a McIlwain tissue chopper as indicated in Figure (2.4). The PFC and NAc core and shell sub-regions were dissected from the first four brain slices and kept in liquid nitrogen until analysis.

2.5.2.1 Tissue preparation

- Please note that the tissue preparation for Western blot analysis was the same for all the proteins (MOR-1, OXR-1 and OXR-2 and orexin peptide A and B). However, the characterisation of antibodies used for each of these proteins was slightly different.

- See the Appendices for the list of buffers, reagents and additional materials used in this protocol.

Tissue samples of each NAc core, NAc shell tissue, PFC and hypothalamus were weighed (grams) and diluted by a factor of 19 using RIPA buffer (Appendix C1). Two µl of protease inhibitor cocktail (Thermo Scientific, Rockford, USA) per 1 ml of RIPA buffer was added to the buffer solution to prevent the loss of proteins from breakdown by enzymes, proteases. After which the tissue samples were sonicated for 10 seconds to assist in protein breakdown and extraction, mixed on a vortex for another 10 seconds and left to stand on ice for 20 minutes.

After 20 minutes, samples were centrifuged at 17200 x g for 30 minutes at 4 °C and the supernatant was collected and kept overnight at -20 °C. The next day, the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA) was used to determine protein concentration (Appendix D1). This was achieved by diluting 10 µl of tissue sample with 50 µl RIPA buffer. Two 25 µl (duplicate) aliquots of each

standard and tissue sample were added to the respective wells of the microtiter plate. This was followed by an addition of 200 µl working reagent (Appendix D1) to each well. The microtiter plate was covered with parafilm and incubated at 37 °C for 30 minutes. After which, the absorbance was recorded at 562 nm using a spectrophotometer (Appendix F).

The absorbance values of the standards were used to generate a standard curve against their known concentration in order to determine the protein concentration (µg/µl) of the tissue samples (Appendix D2). From this, different protein samples (10 µg, 20 µg and 30 µg protein) were loaded into the wells for the characterisation for specific antibodies and Western blot procedures.

2.5.2.2 Characterisation procedure

2.5.2.2.1 Electrophoresis

Characterisation of MOR-1, OXR-1 and OXR-2 as well as orexin A and B protein levels began with testing different concentrations of protein (10 µg, 20 µg and 30 µg) to yield the best optimal results for analysis of protein levels (Figure 2.9). It is important to note that the characterisation of the larger molecular weight proteins (MOR-1, 50 kDa; OXR-1, 56 kDa and OXR-2, 38 kDa) was performed using a 12 % separating and 5 % stacking (bis-acrylamide) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel using Bio-Rad Laboratories (USA) apparatus (Appendix D3). This type of gel contains large pores that are suitable for the separation of larger molecular weight proteins such as MOR-1, OXR-1 and OXR-2. A more concentrated SDS-PAGE gel (18 % separating and 5% stacking) (smaller pore size) was used for the smaller molecular weight peptides (Orexin A, 3.56 kDa and Orexin B, 2.94 kDa)(Appendix D4).

Each of these protein concentrations (10 µg, 20 µg and 30 µg) were loaded onto four gels using a Hamilton syringe which was rinsed well between sample loading. Moreover, a larger molecular weight protein marker (M) for the large proteins (MOR-1, OXR-1 and OXR-2) and smaller molecular weight protein marker for the small proteins (Orexin A and B) was added in the respective wells of the gel as

indicated in Figure 2.9 for estimating the size of the proteins (Appendix D5). Gel electrophoresis was performed at 150 – 200 V for 1.17 hours to 2.5 hours in order to get a good separation of proteins according to molecular weight.

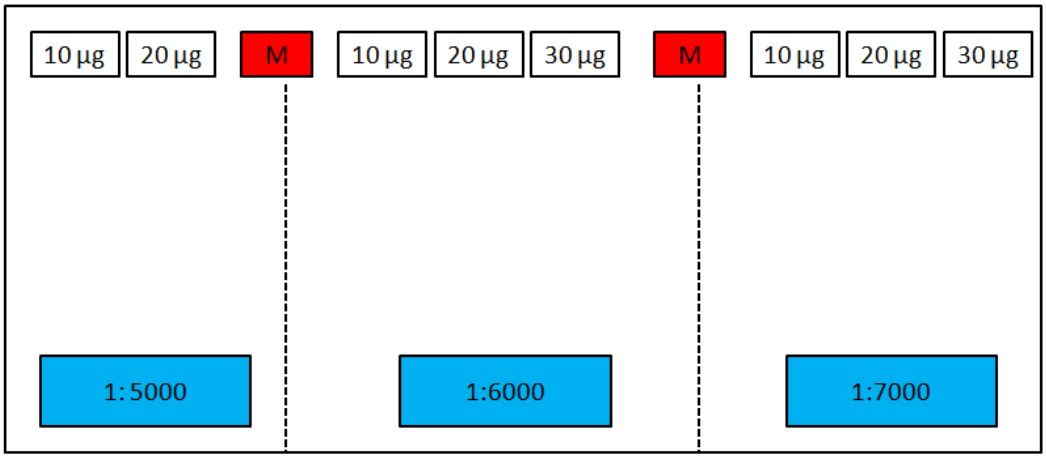


Figure 2.9: An example of the characterisation gel layout. Different amounts of sample tissue protein (µg) were loaded on the gel. The membrane was cut (along the dotted line indicating where the protein marker (M) was loaded) into three parts. Each of the three parts were incubated with different concentrations (1:5000, 1:6000: 1:7000) of the primary antibody.

2.5.2.2.2 Electrotransfer

After gel electrophoresis, the larger proteins (MOR-1, OXR-1 and OXR-2) were transferred into the nitrocellulose membrane (0.45 µm, Hybond ECL, GE Health Life Sciences, UK) at 100 V for 1 hour. Orexin A and B protein samples were transferred using a nitrocellulose membrane with a small pore size (0.2 µm) since a 0.45 µm nitrocellulose membrane increases the risk of losing small protein through the larger pores. The nitrocellulose membranes were stained with Ponceau S to determine if protein transfer was successful. This was followed by a 30-minute wash step (3 x 10 minutes) with distilled water to remove the stain before the blocking agent was added to the membranes.

2.5.2.2.3 Blocking and antibody incubation

A 5 % bovine serum albumin (BSA) in phosphate buffered saline with 0.1 % Tween (PBS-T) solution was used to block the nitrocellulose membrane for 2 hours at room temperature or overnight at 4 °C in order to reduce background. This is achieved by preventing non-specific binding of the primary antibody to the membrane, as it contains a large number of proteins. The different blocking times were tested in order to determine optimal conditions for analysing the protein band of interest. It is important to note that 5% BSA was chosen as the optimal blocking buffer solution after the 5 % non-fat milk buffer solution did not produce any protein bands on the X-ray film, suggesting that the 5 % non-fat milk buffer solution was blocking the signal of the primary antibodies.

The nitrocellulose membranes were then immunolabelled with rabbit polyclonal IgG primary antibody against MOR-1(sc- 7488, Santa Cruz Biotechnology) at different dilution ratios (1:200 - 1:7000) for analysing MOR-1 protein levels (Figure 2.8). A goat polyclonal IgG primary antibody against OXR-1 (sc- 8072, Santa Cruz Biotechnology) and OXR-2 (sc- 8074, Santa Cruz Biotechnology) at different dilution ratios (1:100 - 1:7000) was used for OXR-1 and OXR-2 protein analysis. It is important to note that the different concentrations of the primary antibodies were tested starting with the dilution ratio recommended by the manufacturer.

The incubation periods of tissue samples with the primary antibody also varied (1.5 hours at room temperature or overnight at 4 °C) to determine optimal dilution of the primary antibodies and incubation time for measuring MOR-1, OXR-1 and OXR-2 as well as orexin A and B protein levels. After incubation with the primary antibodies, the membranes were washed for 30 minutes (3 X10 minute) in order to remove excess unbound antibody and minimize background noise. This was followed by incubation with a secondary antibody (Goat-anti Rabbit, conjugated to a horseradish peroxidase (HRP) enzyme (GAR, IgG (H+L) - HRP Conjugate, Bio-Rad #170-6515) for MOR-1 and (Rabbit-anti goat, RAG IgG-HRP Conjugate,sc-2768, #F2910, Santa Cruz Biotechnology) for OXR-1 and OXR-2 to produce a signal corresponding to the position of the target protein. The HRP attached to the secondary uses an oxidising agent, hydrogen peroxide to break down the

chemiluminescent agent in the ECL substrate thus producing photo signal as a by-product (Veitch et al., 2004). Various concentrations (1:5000 - 10000) of GAR (MOR) and RAG (OXR-1 and OXR-2) were tested during the characterisation stage for optimising MOR-1, OXR-1 and OXR-2 as well as orexin A and B measurement. Incubation of the secondary antibody was conducted for 2 hours at room temperature.

2.5.2.2.4 Detection

After incubation with the secondary antibody, excess antibody was removed by washing the nitrocellulose membranes for 30 minutes (3 X10 minute) with PBS-T. After which, 1500 µl Clarity Western ECL Substrate (Bio-Rad Laboratories Inc. USA) consisting of reagent A (750 µl, clarity Western peroxide reagent) and reagent B (750 µl, luminol/enhancer reagent) was applied to the nitrocellulose membranes. The membranes were exposed to the ECL substrate for 1 minute by swirling the clear transparencies containing the membranes. Excess ECL substrate solution was removed. The HRP-conjugate bound to the secondary antibody catalyses the luminal substrate (B) in the presence of substrate A (peroxide) thereby producing a luminescence signal. The luminescence was captured by exposing the membranes to X-ray film. Exposure time to the X-ray film was also optimised.

2.5.2.2.5 Quantification using UN-SCAN IT

All the X-ray films were scanned at 600 dpi and saved in a tag image file format (TIFF). The images were then analysed using the positive segment analysis of the UN-SCAN-IT software (Silk Scientific Inc. Utah, USA). The blot images were converted to a grey scale and a background segment was selected to correct for background pixilation. Bands of interest were selected and pixel density of each protein band was determined (minus background pixel density). The pixel total in each segment and pixel total percentage data was generated by the UN-SCAN-IT software and used to calculate MOR-1, OXR-1 and OXR-2 protein levels. The

protein levels were calculated by dividing the total number of pixels of each protein band by the sum of the pixel totals of all bands in that segment in order to obtain the pixel total of each protein band as a percentage of the pixel total of all the samples on the membrane.

2.5.2.2.6 Optimal conditions for MOR-1, OXR-1 and OXR-2 protein levels

Based on the characterisation work, the best conditions for analysing MOR-1 in the NAc, OXR-1 and OXR-2 protein levels in the PFC are indicated in Table 2.1. A linear curve was generated for determining the optimal protein concentration for each primary antibody and for detecting differences in protein levels between the experimental groups (Figure 2.10). A protein aliquot of 20 µg for MOR-1 and OXR-2 and 24 µg for OXR-1 were chosen using the standard curves shown in Figure 2.9. Protein samples were loaded as indicated in Figure 2.11; different samples (n=8) from the four experimental groups; NMS rats, NMS rats exposed to CCL, MS and MS rats subjected to CCL were loaded as indicated in Figure 2.11. Samples that were not easily quantified due to technical artefacts were repeated. The rest of the Western blot assay analysis was carried out as stated in the characterisation procedure section.

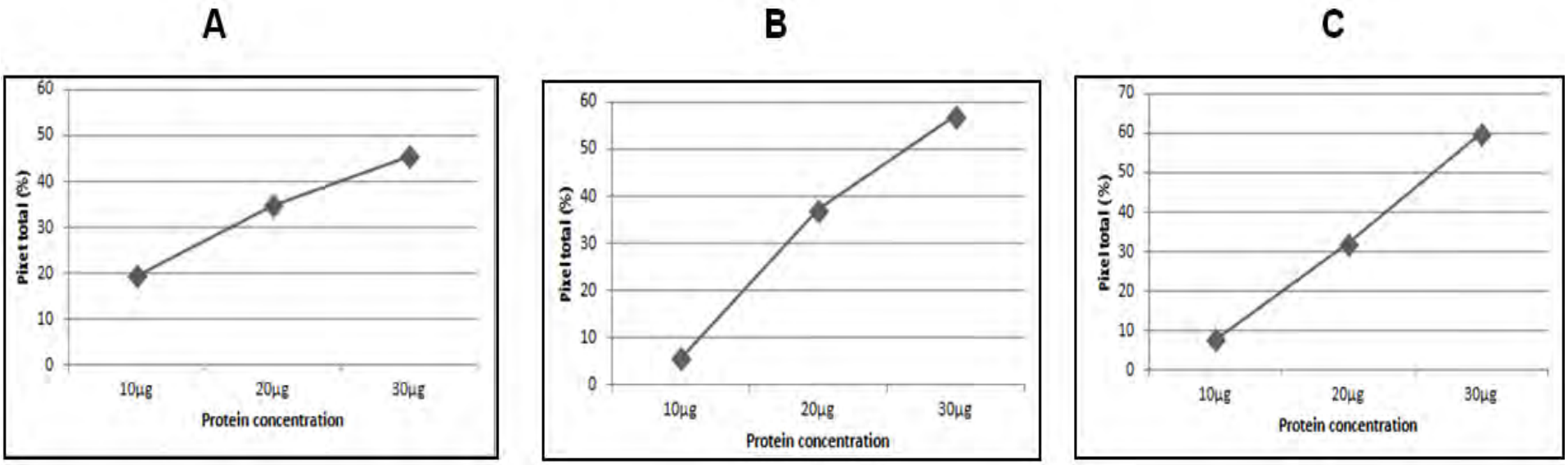


Figure 2.10: Optimal protein concentration graphs. Linear curve graphs used to optimise the amount of protein applied to the gel for MOR-1 (A), OXR-1 (B) and OXR-2 (C) analysis.

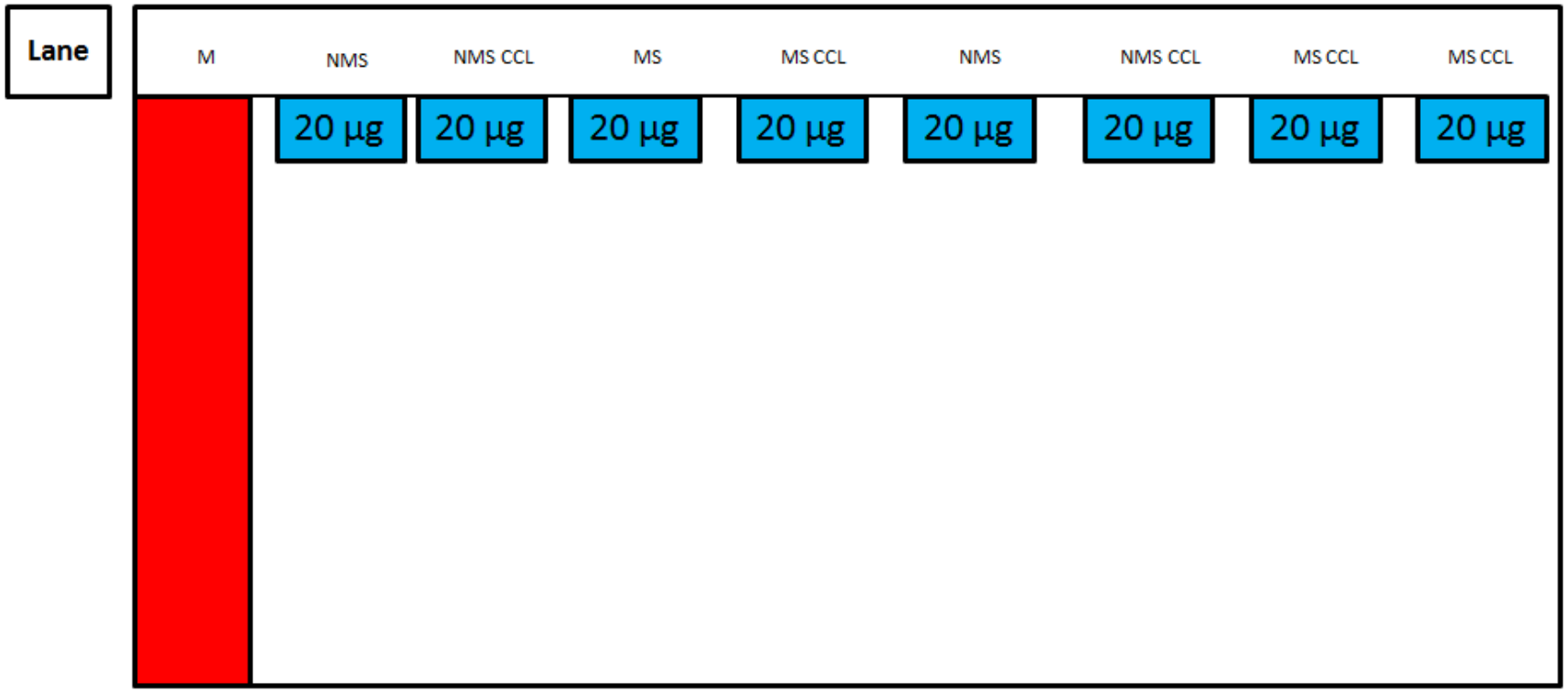


Figure 2.11: An example of a gel layout used for loading protein samples (n=8) in the gel. Two different protein samples from each experimental group: non-maternal separated (NMS) rats, NMS rats exposed to CCL, maternal separated (MS) animals and MS rats subjected to CCL (8 in total) were loaded in the gel. **Abbreviation:** Molecular weight marker (M)

Table 2.1: Optimal conditions for measurement of MOR-1 in the NAc, OXR-1 and OXR-2 in the PFC protein levels

Protein concentration	Gel electrophoresis (150 V)	Transfer (100 V)	Blocking (5 % BSA)	Primary antibody concentration	Secondary antibody concentration (2 Hour)
20 µg	Upto 35 kDa M (≈ 2.5 hours)	1 Hour	2 Hours	1: 6000 (MOR-1) (overnight incubation)	1: 10000 (GAR)
24 µg	1.17 hours	1 Hour	overnight	1: 3000 (OXR-1) (1.5 hours)	1: 10000 (RAG)
20 µg	1.17 hours	1 Hour	overnight	1:4000 (OXR-2) (1.5 hours)	1: 10000 (RAG)

Abbreviations: µ-opioid receptor (MOR-1), Bovine serum albumin (BSA), Goat anti-rabbit (GAR), Kilo Dalton (KDa), Molecular weight protein marker (M), orexin receptor 1 and 2 (OXR-1 and OXR-2), Rabbit anti-goat (RAG), Volts (V)

Table 2.2: Different conditions used to characterise Western blot analysis of orexin A and B peptides in the hypothalamus

Protein Concentration	Gel electrophoresis (200V)	Transfer
10 µg, 20 µg and 30 µg	18 % SDS-PAGE gel	30 minutes and 1 hour at 40, 60, 80 and 100 V
70 µg, 80 µg and 90 µg	30 minute – 1.5 hours running time	Nitrocellulose and PVDF membranes

Abbreviations: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Polyvinylidene difluoride (PVDF), Volts (V)

Characterisation of orexin A and B peptides was unsuccessful with tissue samples of 10 µg, 20 µg and 30 µg proteins. There were no protein bands visible below the15kDa molecular weight protein marker as shown in Figure 2.12. It was therefore proposed that the amount of orexin A and B protein in the tissue samples was too low to be detected using Western blot analysis. To test this hypothesis, the amount of protein applied to the gel was increased to 70 µg, 80 µg and 90 µg, despite this there were still no protein bands present with the molecular weight size of orexin A (3.56 kDa) or Orexin B (2.94 kDa) peptides.

It was also hypothesised that the small peptides might be running off the gel before electrophoresis was completed. Therefore, instead of performing the gel electrophoresis for 1.5 hours, of which was chosen in order to get a clear separation of the protein samples, a shorter electrophoresis time (30 minutes) was tested to determine if this would minimise the loss of small proteins. However, there were still no proteins present between 15 kDa to 1.7 kDa (Figure 2.12). Several attempts were made to optimise the procedure, such as altering the transfer conditions as shown in Table 2.2. For example, high voltage (e.g. 100 V) vs. a low voltage (e.g. 60 V) was tested to for determine the best electric field strength for the transfer of small

proteins to the membrane. Both 100 V and 60 V did not yield any differences, even transferring for different periods of time (1 hour vs. 30 minutes) did not produce any protein bands on the membranes. Similarly, no protein bands were visible when either nitrocellulose or PDVF membrane were used, suggesting that the problem might be due to the small molecular weights of the peptides, A (3.56 kDa) and Orexin B (2.94 kDa). This also suggested that the western blot might not have been sensitive enough to detect these peptides, despite several studies using the same technique to analyse orexin A and B protein levels in the hypothalamus and NAc (Karteris et al., 2005; Zhang et al., 2007). For future studies, more sensitive techniques such as the radioimmunoassay or ELISA assay techniques should be considered for measuring orexin A and B protein levels.

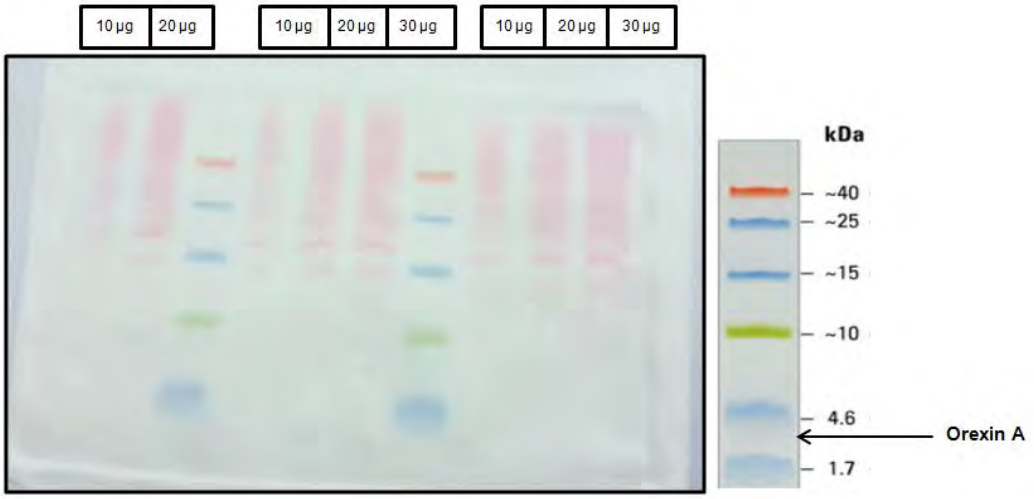


Figure 2.12: Characterisation of orexin peptides. An example a nitrocellulose membrane (0.2 µm) with different amounts of protein samples (10 µg, 20 µg and 30 µg) for orexin A peptide. There were no protein bands visible around the 4.6 KDa molecular weight protein marker to indicate the presence of orexin A (3.56 KDa).

2.5.2.2.7 Housekeeping proteins

The same blots used during the characterisation procedure of the MOR-1, OXR-1 and OXR-2 protein levels were used to characterise the monoclonal anti α-tubulin

antibody produced in mouse (MOR-1) (#T5168, Sigma-Aldrich, Missouri, USA) and anti-p38 MAP Kinase antibody produce in rabbit (OXR-1 and OXR-2) (#M0800, Sigma-Aldrich, Missouri, USA). Both α anti-tubulin and anti-p38 were used as loading controls to confirm that samples were evenly loaded in the gel, as this is important for comparing protein levels between the experimental groups. Characterisation of the α-tubulin antibody was achieved by washing the blots with distilled water for 20 minutes (2 x10 minutes) and followed by a 5-minute exposure to 0.2 M NaOH solution to strip primary and secondary antibodies from the blots (Appendix D6). After the 5-minute stripping period, the blots were washed again with distilled water (2 x 10 minutes). The blots were then blocked with 5 % non-fat milk in PBS-T for 2 hours at room temperature. The remaining steps of the Western blot assay were performed in the same way as described in the characterisation procedure section except that goat anti-mouse (GAM, IgG (H+L) - HRP Conjugate, Bio-Rad #172-1011) secondary antibody was used during the incubation step.

Table 2.3: Optimal conditions for primary and secondary antibodies used to measure α- tubulin and p38 in the different gels

Primary Antibody	Secondary Antibody
1: 6000 (α-tubulin for MOR-1)	1: 10 000 (GAM)
1 : 8000 (p38 for OXR-1)	1: 10 000 (GAR)
1: 8000 (p38 for OXR-2)	1: 10 000 (GAR)

Abbreviations: μ-opioid receptor (MOR-1), orexin receptor 1 and 2 (OXR-1 and OXR-2), goat anti mouse (GAM), goat anti rabbit (GAR)

2.6 Statistical Analysis

Data analysis was performed using Statistica version 11 (Statsoftinc, Tulsa, Oklahoma, USA) and graphs were designed using the GraphPad Prism version 6 (GraphPad software, San Diego, California, USA) software programme. The

Shapiro-Wilk test was used to determine if the data were normally distributed. Glutamate- and potassium-stimulated [^{3}H]DA release data, 5-HT levels and MOR-1 levels data were all normally distributed. Similarly, the pixel total percentages of OXR-1 and OXR-2 levels data were normally distributed. However, 5-HT levels in the PFC were not normally distributed. Similarly, when the OXR-1 protein from each sample was normalised against the housekeeping protein (p38), the data were not normally distributed. Furthermore, p38 protein levels (for OXR-1) were also not normally distributed.

For all the normally distributed data, a parametric analysis was conducted using a two-way analysis of variance (ANOVA) to determine statistical significance of the effects of MS and CCL on the experimental groups; non-maternally separated (NMS) rats, NMS rats subjected to CCL (NMS CCL), MS rats and MS rats subjected to CCL (MS CCL). Two-way ANOVA was followed by Duncan's post hoc test. All the data are presented as mean and standard error mean (SEM).

On the other hand, non-parametric data were analysed using the Kruskal-Wallis test to determine statistically significant differences in 5-HT levels in the PFC between the experimental groups. Where appropriate, significant Kruskal-Wallis tests were followed by Dunn's post hoc test. Data are expressed as median and interquartile range (IQR).

CHAPTER 3: RESULTS

3.1 Glutamate- and potassium-stimulated [^{3}H]DA release in the NAc core

Two-way ANOVA of glutamate-stimulated [^{3}H]DA release revealed that there was no significant effect of MS ($F_{(1,49)}$ =1.564, p=0.217) or CCL ($F_{(1,49)}$ =0.704, p=0.406) in the NAc core (Figure 3.1). There was no interaction between the main effects of MS and CCL (MS*CCL; $F_{(1,49)}$ =1.558, p=0.218) on glutamate-stimulated [^{3}H]DA release in the NAc core.

Two-way ANOVA of potassium-stimulated [^{3}H]DA release did not show any significant effect of MS ($F_{(1,43)}$=0.659, p=0.421) or CCL ($F_{(1,43)}$=0.107, p=0.746) in the NAc core (Figure 3.2). There was also no interaction between MS and CCL (MS*CCL; $F_{(1,43)}$ =1.321, p=0.257) on potassium-stimulated [^{3}H]DA release in the NAc core.

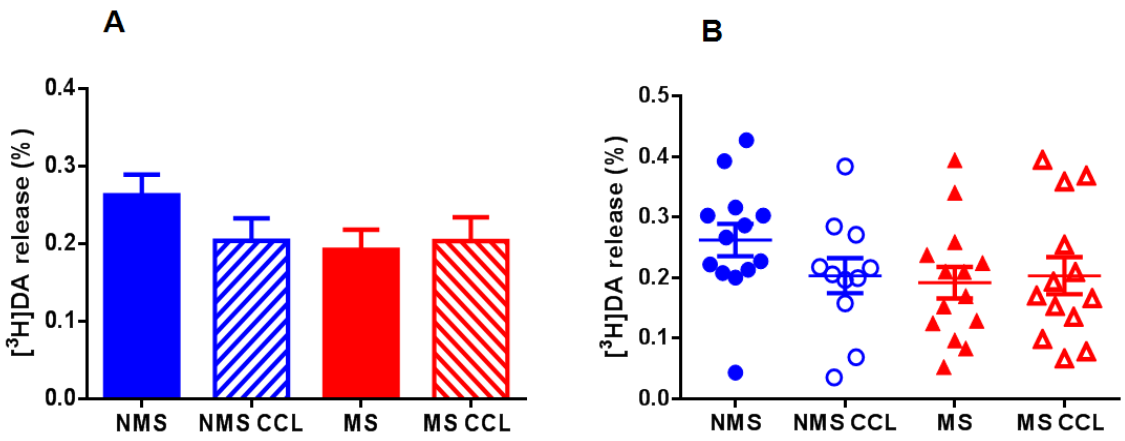


Figure 3.1: Column (A) and scatter plot (B) graphs showing glutamate-stimulated [^{3}H]DA release measured in the NAc core at P80 using in vitro superfusion. Two-way ANOVA showed no significant differences in glutamate-stimulated [^{3}H]DA release between the groups; non-maternally separated animals (NMS, n=13); non-maternally separated animals subjected to chronic constant light (NMS CCL, n=11); maternally separated animals (MS, n=14) and maternally separated animals exposed to chronic constant light (MS CCL, n=13). Data presented as mean ± SEM.

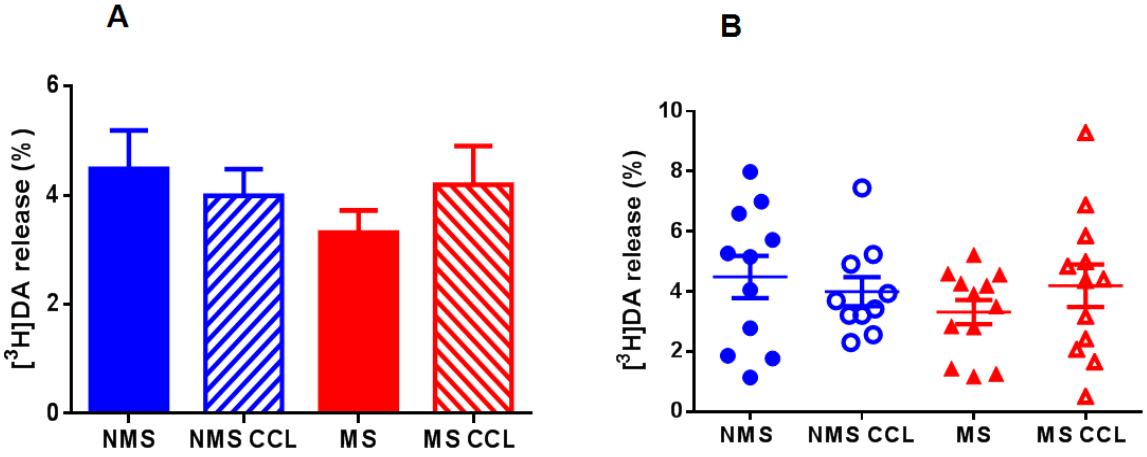


Figure 3.2: Column (A) and scatter plot (B) graphs for potassium-stimulated [^{3}H]DA release in the NAc core at P80. Two-way ANOVA indicated no significant differences in potassium-stimulated [^{3}H]DA release between experimental groups; non-maternally separated animals (NMS, n=11), non-maternally separated animals exposed to chronic constant light (NMS CCL, n=10), maternally separated (MS, n=12) and maternally separated animals exposed to chronic constant light (MS CCL, n=12). Data presented as mean ± SEM.

3.2 Glutamate- and potassium-stimulated [^{3}H]DA release in the NAc shell

Two-way ANOVA of glutamate-stimulated [^{3}H]DA release did not reveal any MS effect in the NAc shell ($F_{(1,46)}$ =2.346, p=0.133). There was no interaction between MS and CCL (MS*CCL; $F_{(1,46)}$ =0.185, p=0.669) on glutamate-stimulated [^{3}H]DA release in the NAc shell. On the other hand, a decreased trend towards significance was observed for CCL exposure ($F_{(1,46)}$ =2.990, p=0.091) on glutamate-stimulated [^{3}H]DA release in the NAc shell (Figure 3.3).

There was also no MS effect found on potassium-stimulated [^{3}H]DA release in the NAc shell (Two-way ANOVA, $F_{(1,38)}$=1.735, p=0.196). However, a trend towards a significant decrease was revealed in potassium-stimulated [^{3}H]DA release after CCL exposure ($F_{(1,38)}$ =3.604, p=0.066) (Figure 3.4). There was also no interaction between the MS and CCL effects on potassium-stimulated [^{3}H]DA release in the NAc shell ($F_{(1,38)}$=0.642, p=0.428).

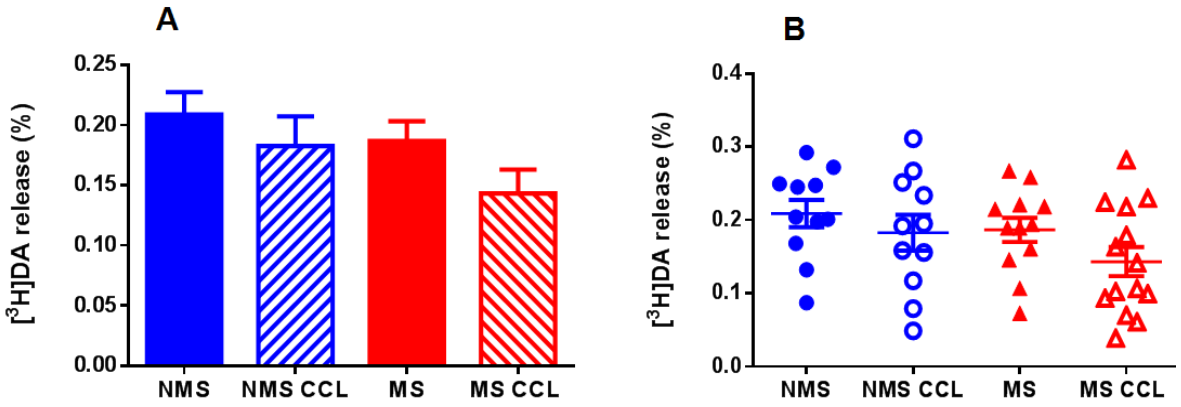


Figure 3.3: Column (A) and scatter plot (B) graphs for glutamate-stimulated [^{3}H]DA release in the NAc shell at P80. No significant differences were observed in glutamate stimulated [^{3}H]DA release between the groups; non maternally separated animals (NMS, n=11); non-maternally separated animals subjected to chronic constant light (NMS CCL, n=11); maternally separated animals (MS, n=12) and maternally separated animals exposed

to chronic constant light (MS CCL, n=14) (Two-way ANOVA). Data presented as mean ± SEM.

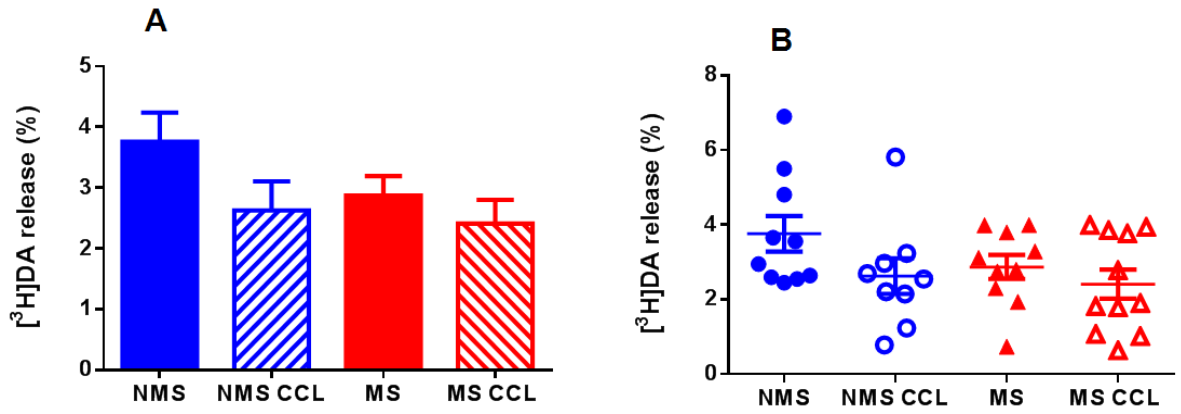


Figure 3.4: Column (A) and scatter plot (B) graphs for potassium-stimulated [^{3}H]DA release in the NAc shell at P80. Two-way ANOVA revealed that there are no significant differences in [^{3}H]DA release after potassium stimulation between the experimental groups; non-maternally separated (NMS, n=10), non-maternally separated animals exposed to chronic constant light (NMS CCL, n=9), maternally separated (MS, n=10) and maternally separated animals exposed to chronic constant light (MS CCL, n=11). Data presented as mean ± SEM.

3.3 Glutamate- and potassium-stimulated [^{3}H]DA release in the NAc

The NAc core and shell data were pooled and the average for each rat used as a representative measure of the function of the NAc as a whole. It is important to note that rats in which only the core or shell data points were obtained, the single core or shell data point was included in the analysis. A decreased glutamate-stimulated [^{3}H]DA release in the NAc was found after MS exposure (Two-way ANOVA, $F_{(1,51)}$ =4.934, p=0.031). On the other hand, there was no main effect of CCL on glutamate-stimulated [^{3}H]DA release, however a decreased trend was observed (Two-way ANOVA, $F_{(1,51)}$=2.863, p=0.097) (Figure 3.5). There was no interaction between MS and CCL (MS*CCL; $F_{(1,51)}$ =0.518, p=0.475) on glutamate-stimulated [^{3}H]DA release in the NAc.

There was neither MS effect (Two-way ANOVA, $F_{(1,43)}$= 1.146, p=0.291) or CCL effect (Two-way ANOVA, $F_{(1,43)}$= 0.068, p=0.795) on potassium-stimulated [^{3}H]DA release in the NAc (Figure 3.6). There was no interaction between MS and CCL (MS*CCL; $F_{(1,43)}$ =0.905, p=0.347) on potassium-stimulated [^{3}H]DA release in the NAc.

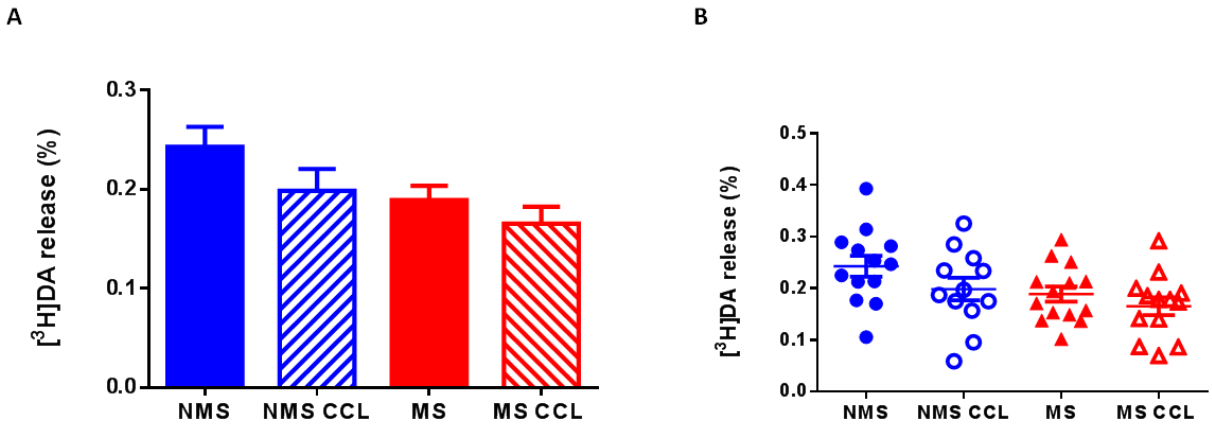


Figure 3.5: Column (A) and scatter plot (B) graphs showing glutamate-stimulated [^{3}H]DA release in the NAc as a whole at P80. Two-way ANOVA indicated a significant effect of MS on glutamate-stimulated [^{3}H]DA release in the NAC between the experimental groups; non-maternally separated (NMS, n=13); non-maternally separated animals subjected to chronic constant light (NMS CCL, n=12); maternally separated (MS, n=14) and maternally separated animals exposed with chronic constant light (MS CCL, n=14).

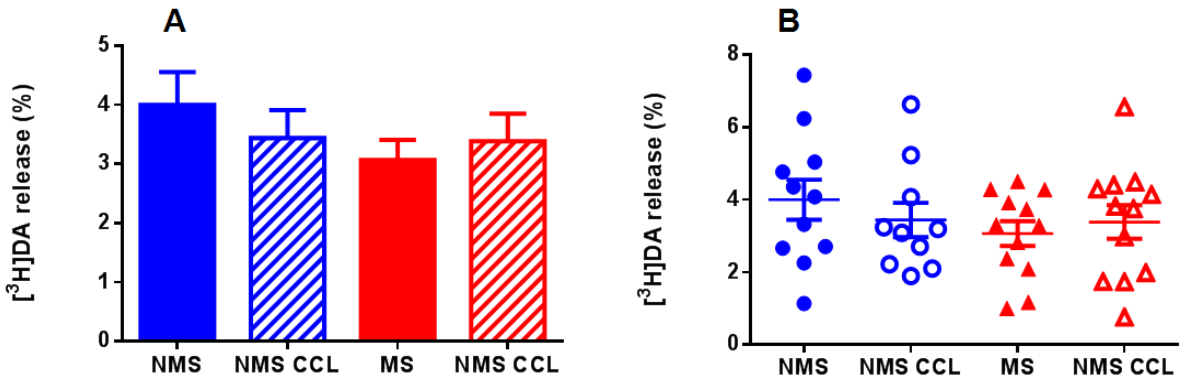


Figure 3.6: Column (A) and scatter plot (B) graphs showing potassium-stimulated [^{3}H]DA release in the NAc as a whole at P80. Two-way ANOVA revealed no significant differences in potassium-stimulated [^{3}H]DA release between the groups; non-maternally separated animals (NMS, n=11); non-maternally separated animals exposed to chronic constant light (NMS, n=10); maternally separated animals (MS, n=12) and maternally

separated animals exposed to chronic constant light (MS CCL, n=12). Data expressed as mean ± SEM.

3.4 Serotonin concentration in the hypothalamus

MS did not reveal any significant effect on 5-HT levels in the hypothalamus (Two-way ANOVA, $F_{(1,37)}$=2.431, p=0.128). There was also no interaction between MS and CCL (MS*CCL; $F_{(1,37)}$ =1.011, p=0.322) on 5-HT levels in the hypothalamus. On the contrary, 5-HT levels were significantly increased in both NMS and MS rats subjected to CCL compared to NMS and MS rats without CCL exposure (Two-way ANOVA, $F_{(1,37)}$=11.810, p=0.002) (Figure 3.7).

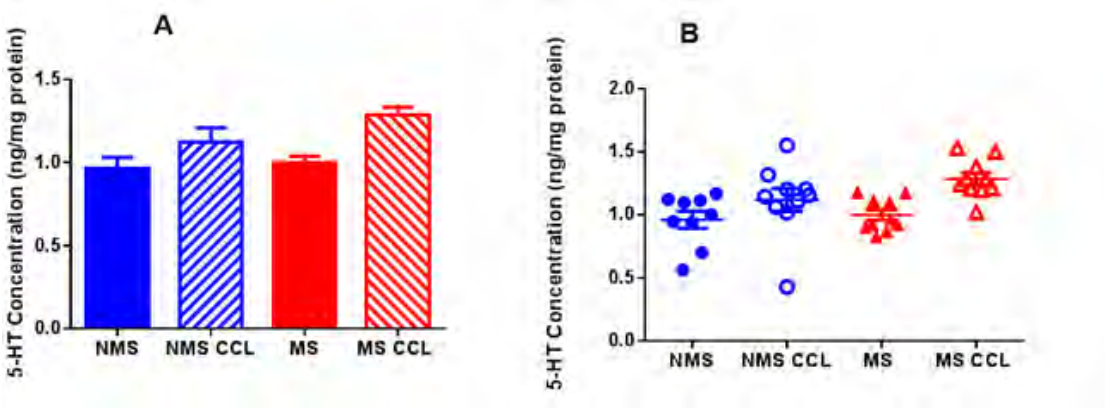


Figure 3.7: Column (A) and scatter plot (B) graphs showing 5-HT concentration (ng/mg) in the hypothalamus at P80. Two-way ANOVA revealed significant effect of CCL on 5-HT in the hypothalamus between the experimental groups; non-maternally separated animals (NMS, n=9); non-maternally separated animals subjected to chronic constant light (NMS CCL, n=10); maternally separated animals (MS, n=10) and maternally separated animals exposed to chronic constant light (MS CCL, n=10).

3.5 Serotonin concentration in the PFC

The Kruskal-Wallis test ($H_{(3,\ 35)}$=20.744, p<0.001) followed by Dunn's multiple comparison test showed that NMS CCL rats had significantly decreased 5-HT levels when compared to NMS (p=0.003). In addition, MS CCL rats had reduced 5-HT levels in comparison to NMS rats (p< 0.001) (Figure 3.8). Furthermore, MS CCL rats had reduced 5-HT concentration when compared to MS, however this did not reach a statistical significance (p=0.058).

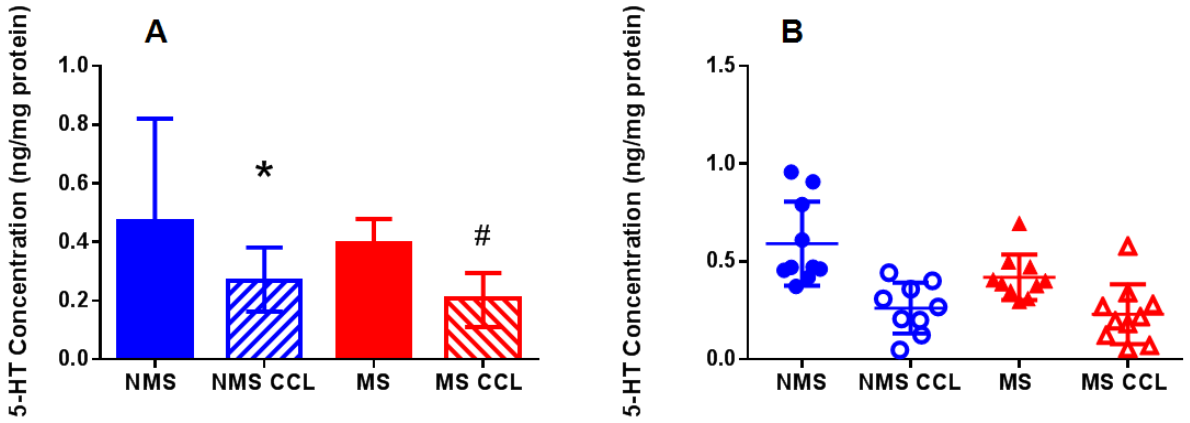


Figure 3.8: Column (A) and scatter plot (B) graphs for 5-HT concentration (ng/mg) measured in the PFC at P80. Kruskal-Wallis test showed significant differences in 5-HT between the experimental groups; non-maternally separated animals (NMS, n=10); non-maternally separated animals exposed to chronic constant light (NMS CCL, n=9); maternally separated animals (MS, n=10) and maternally separated animals exposed to chronic constant light (MS CCL, n=10). *NMS CCL rats had decreased 5-HT compared to NMS rats, p< 0.05. #MS CCL rats had significantly decreased 5-HT compared to NMS, p<0.001 (Dunn's multiple comparison test). Data expressed as median and IQR.

3.6 MOR-1 protein levels in the NAc core

Neither MS (Two-way ANOVA, $F_{(1,35)}$ =0.453, p=0.505) nor CCL (Two-way ANOVA, $F_{(1,35)}$=0.931, p=0.341) had an effect on MOR-1 protein levels in the NAc core (Figure 3.9). There was no interaction between MS and CCL (MS*CCL; $F_{(1,35)}$ =2.245, p=0.143) on MOR-1 protein levels in the NAc core. Two-way ANOVA of MOR-1 protein levels when normalised did not reveal neither a MS ($F_{(1,35)}$=0.486,

p=0.491) nor a CCL ($F_{(1,35)}$=0.168, p=0.685) effect on MOR-1 protein levels in the NAc core (Appendix H4). There was no interaction between MS and CCL (MS*CCL; $F_{(1,35)}$ =1.171, p=0.287) on normalised MOR-1 protein levels in the NAc core.

Two-way ANOVA of α-tubulin protein levels revealed no significant effect of MS ($F_{(1,35)}$=0.069, p=0.794) or CCL ($F_{(1,35)}$=1.045, p=0.314) or interaction between MS and CCL (MS*CCL; $F_{(1,35)}$ =0.552, p=0.462) for α-tubulin protein levels, suggesting that samples were loaded equally (Appendix H4).

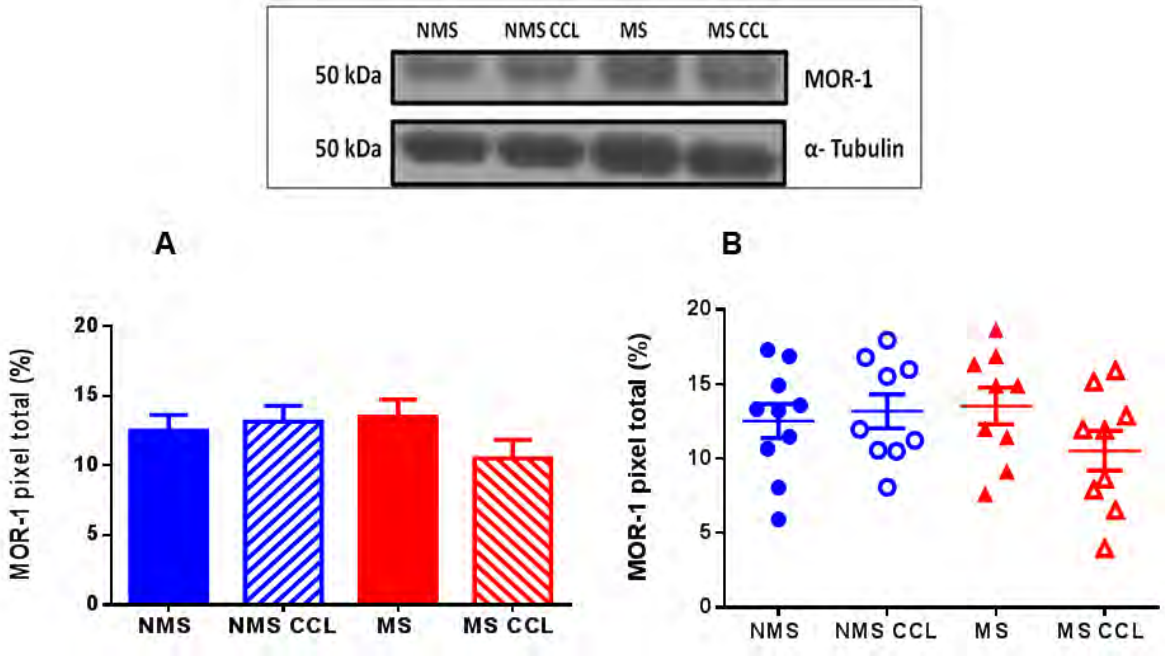


Figure 3.9: Column (A) and scatter plot (B) graphs showing μ-opioid receptor (MOR-1) protein levels as measured by western blot analysis of the NAc core. No significant differences were found between the groups: non-maternally separated animals (NMS, n=10); maternally separated animals exposed to chronic constant light (NMS CCL, n=9), maternally separated animals (MS, n=9) and maternally separated animals exposed to chronic constant light (MS CCL, n=9) (Two-way ANOVA). Data represented as mean ± SEM.

3.7 MOR-1 protein levels in the NAc shell

There was no effect MS (Two-way ANOVA, $F_{(1,32)}$ =0.132, p=0.719) or CCL exposure (Two-way ANOVA, $F_{(1,32)}$=0.057, p=0.813) on MOR-1 levels in the NAc shell (Figure 3.10). There was no interaction between MS and CCL (MS*CCL; $F_{(1,32)}$ =1.252, p=0.272) on MOR-1 protein levels in the NAc shell.

Two-way ANOVA of MOR-1 protein levels did not reveal any MS ($F_{(1,32)}$=0.132, p=0.719) or CCL ($F_{(1,32)}$=0.057, p=0.813) effects on MOR-1 proteins levels in the NAc shell when normalised against α-tubulin (Appendix H5). There was also no interaction between MS and CCL (MS*CCL; $F_{(1,32)}$ =1.263, p=0.269) when MOR-1 protein levels were normalised in the NAc shell.

Furthermore, two-way ANOVA of α-tubulin protein levels revealed no significant effect in either MS ($F_{(1,32)}$=0.363, p=0.552) or CCL ($F_{(1,32)}$=1.824, p=0.187) exposure on α-tubulin protein levels, thus indicating equal loading of samples (Appendix H5). There was no interaction between the main effects of MS and CCL (MS*CCL; $F_{(1,32)}$ =1.069, p=0.309) on α-tubulin protein levels.

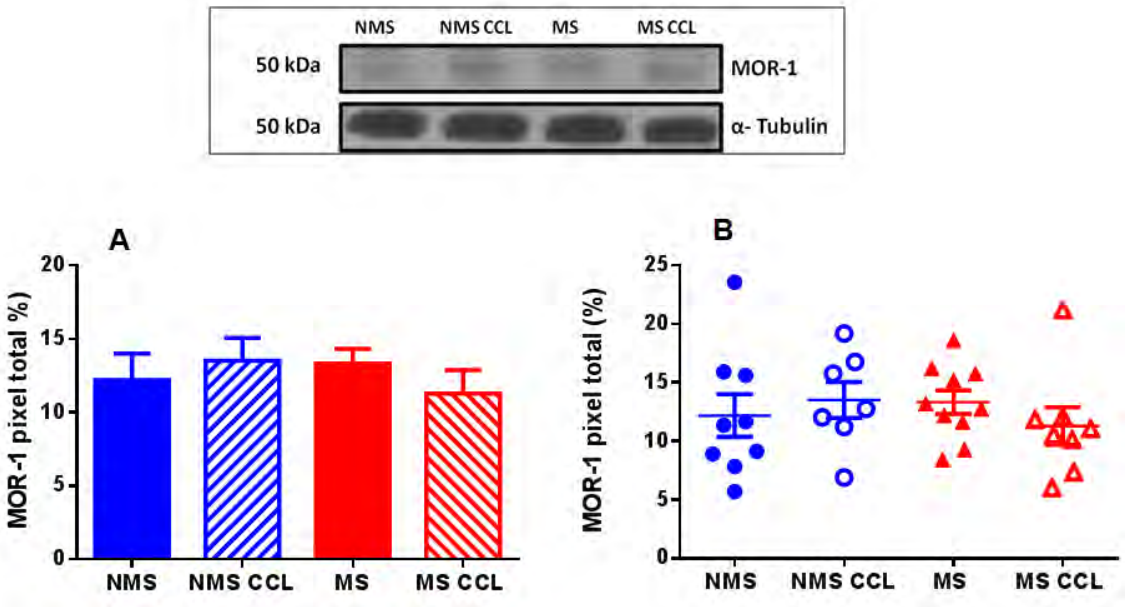


Figure 3.10: Column (A) and scatter plot (B) graphs of μ-opioid receptor (MOR-1) protein levels measured by western blot analysis of the NAc shell. No significant differences were found between the groups: non-maternally separated animals (NMS, n=9); non-maternally separated animals exposed to chronic constant light (NMS CCL, n=7), maternally separated animals (MS, n=10) and maternally separated animals exposed to chronic constant light (MS CCL, n=8) (Two-way ANOVA). Data represented as mean ± SEM.

3.8 MOR-1 protein levels in the NAc

The MOR-1 protein levels in the NAc shell and core were averaged and pooled to obtain a representative measure of the function of the NAc as a whole. In cases where either the shell or core data were not available, only the shell or core result was used for statistical analysis. There was also no significant effect of either MS (Two-way ANOVA, $F_{(1,43)}$ =1.530, p=0.223) or CCL (Two-way ANOVA, $F_{(1,43)}$=0.013, p=0.909) on MOR-1 levels in the NAc (Figure 3.11). There was no interaction between MS and CCL (MS*CCL; $F_{(1,43)}$ =1.288, p=0.2629) on MOR-1 protein levels in the NAc.

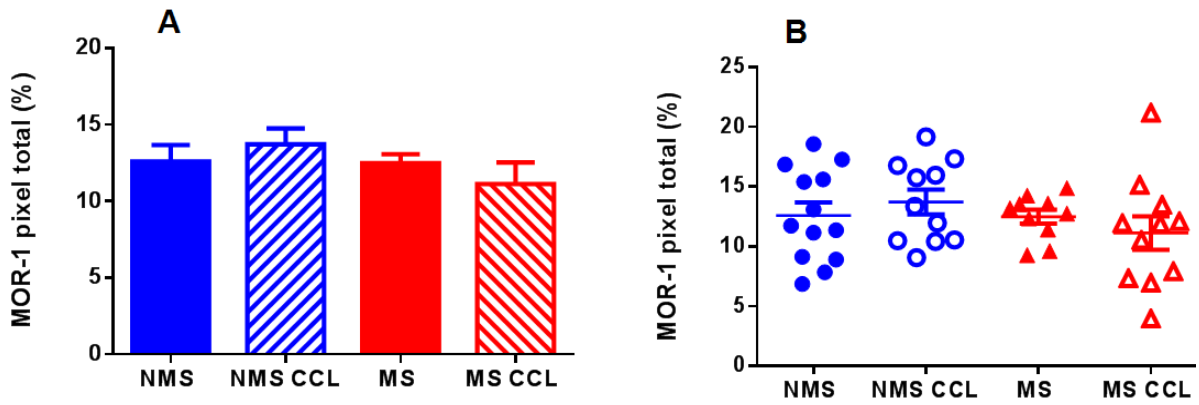


Figure 3.11: Column (A) and scatter plot (B) graphs showing μ-opioid receptor (MOR-1) protein levels measured by western blot analysis of the NAc. No significant differences were found between the groups: non-maternally separated animals (NMS, n=13); non-maternally separated animals treated with chronic constant subjected to chronic constant light (MS CCL, n=11), maternally separated rats (MS, n=10) and maternally separated rats subjected to chronic constant light (MS CCL, n=11) (Two-way ANOVA). Data represented as mean ± SEM.

3.9 OXR-1 protein levels in the PFC

Two-way ANOVA of OXR-1 protein levels revealed a significant interaction between MS and CCL on OXR-1 protein levels ($F_{(1,36)}$ = 8.303, p=0.007), whereby CCL had an opposing effect in NMS and MS rats. Duncan's post hoc test revealed that MS rats had significantly increased OXR-1 protein levels when compared to NMS rats (p=0.032) and MS CCL rats (p=0.035) (Figure 3.12). Similarly, when the OXR-1 protein from each sample was normalised against the housekeeping protein (p38), the data were not normally distributed and the Kruskal-Wallis test ($H_{(3, 34)}$=10.090, p=0.018) revealed that there was a significant difference in OXR-1 protein levels between groups. Dunn's multiple comparison test revealed that MS rats had significantly increased OXR-1 protein levels when compared to NMS rats (p=0.020) (Appendix H6). Furthermore, there were no significant differences in p38 protein levels between the experimental groups, thus indicating equal loading of samples, Kruskal-Wallis test ($H_{(3, 34)}$=0.544, p=0.909) (Appendix H6).

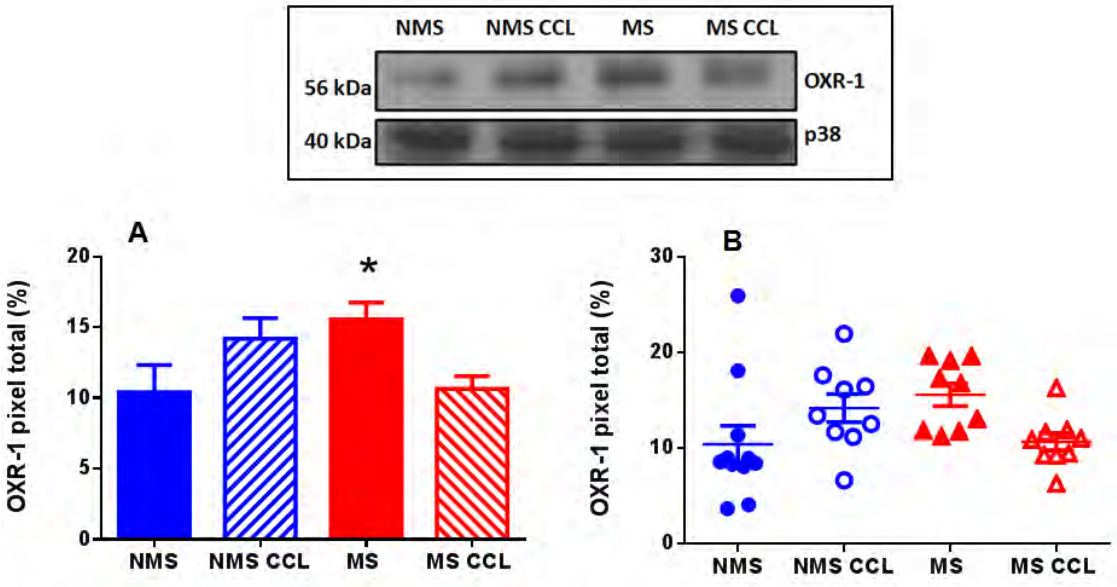


Figure 3.12: Column (A) and scatter plot (B) graphs showing orexin receptor 1 (OXR-1) protein levels as measured by western blot analysis of the PFC. CCL had an opposite effect in NMS and MS rats as indicated by the significant interaction between MS and CCL in OXR-1 protein levels between the experimental groups; non- maternally separated animals (NMS, n=11); non-maternally separated animals subjected to chronic constant light (NMS CCL, n=9), maternally separated animals MS (n=9) and MS CCL (n=9) (Two-way ANOVA). *MS rats showed a significant increase in OXR-1 protein levels compared to NMS rats (p=0.032) and MS CCL rats (p=0.035), Duncan's post hoc test. Data are represented as mean ± SEM.

3.10 OXR-2 protein levels in the PFC

There was no effect MS (Two-way ANOVA, $F_{(1,37)}$ =0.906, p=0.348) or CCL exposure (Two-way ANOVA, $F_{(1,37)}$=0.412, p=0.525) on OXR-2 levels in the PFC when only the pixel total (%) was analysed (Figure 3.13.1). However, a trend towards a significance between MS and CCL (MS*CCL; $F_{(1,37)}$ =3.791, p=0.059) was found in OXR-2 protein levels. When the OXR-2 protein levels were normalised an interaction between MS and CCL (MS*CCL; $F_{(1,37)}$ =9.391, p=0.004) was revealed. Whereby, CCL had an opposing effect in NMS and MS rats, in which OXR-2 protein levels in NMS rats were increased by CCL exposure and the opposite was found in MS rats. Duncan's post hoc analysis revealed that MS rats had increased OXR-2 protein levels compared to NMS (p=0.010) and MS CCL

rats (p=0.043) (Appendix H7). Moreover, NMS CCL rats had increased OXR-2 protein levels when compared to NMS rats; however this did not reach statistical significance (p=0.051).

Furthermore, two-way ANOVA of p38 protein levels revealed no significant effect in either MS ($F_{(1,37)}$=0.056, p=0.815) or CCL ($F_{(1,37)}$=1.436, p=0.239) exposure, thus indicating equal loading of samples (Appendix H7). There was also no interaction between MS and CCL (MS*CCL; $F_{(1,37)}$ =1.308, p=0.260) on p38 protein levels.

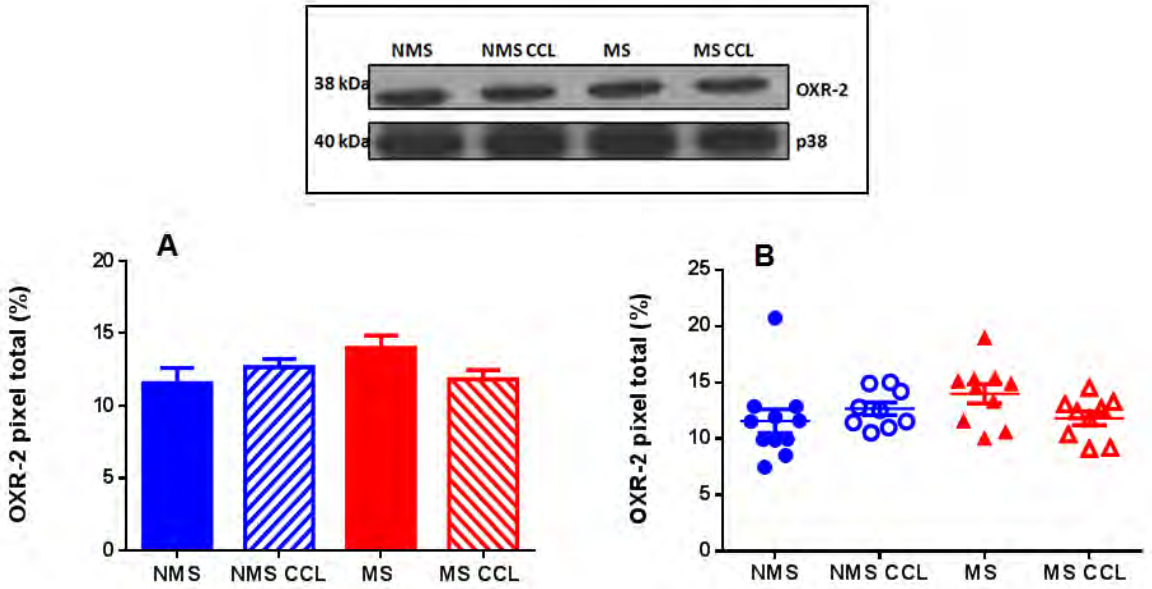


Figure 3.13.1: Column (A) and scatter plot (B) graphs of orexin receptor 2 (OXR-2) protein levels as measured by western blot analysis of the PFC. Two-way ANOVA did not show any significant differences in OXR-2 levels when only the pixel total % was analysed between the experimental groups; non-maternally separated animals NMS (n=11); non-maternally separated animals treated with chronic constant light (NMS CCL, n=9), maternally separated animals (MS, n=10) and maternally separated animals subjected with chronic constant light (MS CCL, n=9). Data represented as mean ± SEM.

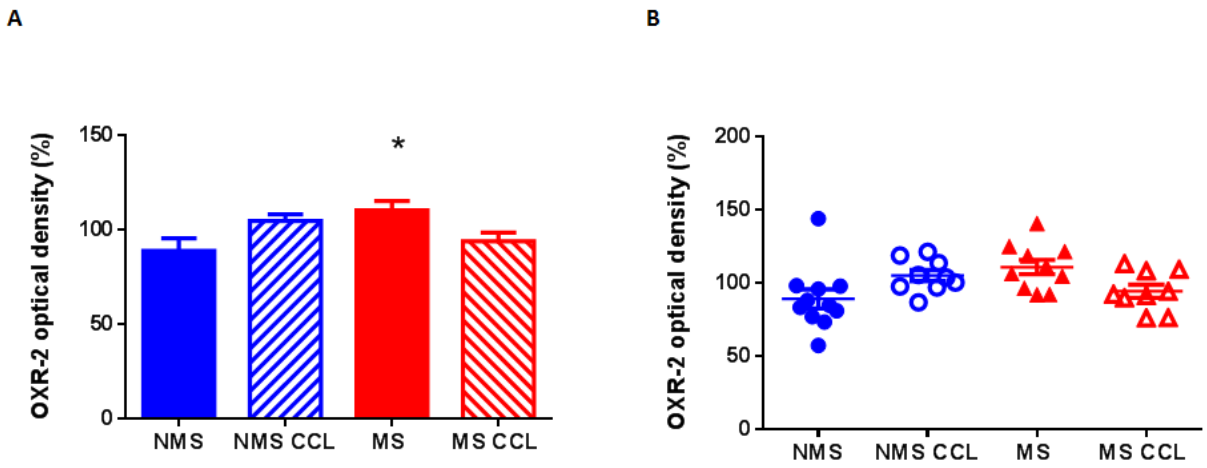


Figure 3.13.2: Column (A) and scatter plot (B) graphs of normalised orexin receptor 2 (OXR-2) protein levels as measured by western blot analysis in the PFC. CCL had an opposite effect in NMS and MS rats as indicated by the significant interaction between MS and CCL in OXR-2 protein levels between the experimental groups; non- maternally separated animals (NMS, n=11); non-maternally separated animals subjected to chronic constant light (NMS CCL, n=9), maternally separated animals MS (n=10) and MS CCL (n=9) (Two-way ANOVA). *MS rats showed a significant increase in OXR-2 protein levels compared to NMS rats (p=0.010) and MS CCL rats (p=0.043), Duncan's post hoc test. Data are represented as mean ± SEM.

CHAPTER 4: DISCUSSION

4.1 CCL exacerbates the reduction of [^{3}H]DA release in the NAc of MS rats

In this study it was found that neither MS or CCL had any effect on glutamate-and potassium-stimulated [^{3}H]DA release in the NAc core. However, in the NAc shell, CCL in combination with either NMS or MS resulted in a trend towards a decrease in [^{3}H]DA release in response to both glutamate- and potassium-stimulation. When the NAc core and shell data were pooled and the average for each rat was used as a measure of the function of the NAc as a whole, there was a significant MS effect. Rats subjected to MS displayed an overall decrease in glutamate-stimulated [^{3}H]DA release in the NAc compared to the NMS rats.

In accordance with these findings, several other rat studies have also reported reduced DA activity in the NAc using different stress paradigms such as the FST and unavoidable electrical shock (Gambarana et al., 1999; Scheggi et al., 2002). For example, decreased levels of extracellular DA in the NAc shell were associated with decreased escape latencies in a shock-escape paradigm (Scheggi et al., 2002). Similarly, Gambarana et al. (1999) conducted a study in which rats were subjected to acute restraint stress and subsequently electric shocks (I mA x 5s) as part of a pretest. For the second part of the study, the same rats that were exposed to pretest and test trials were tested (48 hours later) again in a chronic stress model. Chronic stress exposure caused a significant decrease in DA levels in the NAc shell in stressed rats compared to control rats. These findings and those reported in the current study indicate that additional stressors (i.e. CCL) have a significant reduction in DA release in the NAc.

Furthermore, Gambarana et al. (1999) administered cocaine and d-amphetamine in stressed and control rats to determine if the decreased DA levels were due to either reduced function of the dopaminergic neurons or enhanced removal from the synaptic cleft by the DA transporters. It was found that DA output was lower in stressed rats compared to control animals after cocaine administration. On the

other hand, there was no difference in DA output after d-amphetamine between the groups. This led to the authors to suggest that reduced function in dopaminergic neurons might be a possible mechanism underlying the reduced DA levels in stressed rats (Gambarana et al., 1999). Thus it is possible that exposure to MS and CCL might have reduced dopaminergic neurons function causing a reduction of [^{3}H]DA release in the nucleus accumbens, future studies are required to validate this notion.

In contrast to the altered DA levels following stress, Jahng et al. (2010) did not report any significant differences in accumbal DA levels of rats subjected to MS and subsequent restraint stress (1 h) compared to control animals. It was proposed that the lack of significant decrease in DA levels following restraint stress was due to long-term effects of MS that might have suppressed the effect of subsequent restraint stress on DA activity in the NAc. Thus the suppressed DA function might have impaired the ability of the DA system to respond to stressful stimuli later in life. This study suggests that although MS alone is not a robust model, it does have a long-term effect on DA function. Further evidence comes from findings reported in the current study; MS alone was not sufficient to cause a significant reduction in [^{3}H]DA release. However, when MS and CCL were combined glutamate- and potassium-stimulated [^{3}H]DA release showed a trend towards a decrease in the NAc shell, suggesting that MS might have suppressed DA activity thereby causing a further reduction in [^{3}H]DA release as a result of exposure to CCL. Therefore, these results provide evidence for the combined effects of MS and CCL in altering DA activity and most importantly as a robust model to study biological systems implicated in depression.

The NAc shell appeared to be more sensitive to the effects of MS and CCL than the NAc core, as there was a tendency for CCL to decrease the release of glutamate- and potassium-stimulated [^{3}H]DA release in the NAc shell. No significant change was noted in the NAc core. A possible explanation for the sensitivity of the shell to the combined effects of MS and CCL might be due to its anatomical connections. The shell is mostly connected to limbic nuclei in the brain whereas the core is mostly connected to the motor nuclei (Heimer et al., 1991). Moreover, the shell has a higher concentration of DA than the core, making it an

important subregion mediating certain DA functions (Heimer et al., 1991). Therefore, the shell is more involved in regulating mood behaviours, hence the altered responsivity of the NAc shell to the effects of MS and CCL compared to the core subregion.

Another plausible explanation is that the combination of MS and CCL might have altered glutamate receptor sensitivity thus reducing [^{3}H]DA release in the NAc. For example, Hu et al.(1999) using in vivo microdialysis showed that group two metabotropic glutamate receptor (mGLuR2/3) agonist reduces extracellular glutamate in the NAc. This means that reduced extracellular glutamate due to enhanced mGLuR2/3 would reduce DA levels in the NAc. Similarly, Xi et al. (2002) found that mGLuR2/3 immunoproteins were highly expressed in the shell and core subregions of the NAc. Moreover, it was reported that mGLuR2/3 agonist caused a dose-dependent decrease in extracellular glutamate levels in the NAc and this effect was reversed by administration of a mGLuR2/3 antagonist (Xi et al., 2002). Also in agreement with these findings, Karasawa et al. (2006) showed that injection of a selective mGLu 2/3 receptor agonist decreased DA levels in the NAc shell whereas an mGLu 2/3 receptor antagonist had opposing effects on DA. Furthermore, increased DA levels induced by an mGLuR2/3 receptor antagonist were shown to induce antidepressant-like behaviour in rats (Chaki et al., 2004). Therefore, the decreased glutamate-stimulated [^{3}H]DA release found in the NAc of MS CCL rats suggests that the effects of MS and CCL might have increased mGLu 2/3 receptor sensitivity in the NAc. This may in turn have reduced the extracellular glutamate levels leading to a subsequent decrease in [^{3}H]DA release. Future studies are required to validate these claims.

The interaction of DA and serotonin at the accumbal level could also be an explanation for the decreased glutamate-stimulated [^{3}H]DA release. Serotonin modulates DA release in the NAc and PFC (Yoshimoto et al., 1996; Hallbus et al., 1997; Di Matteo et al., 2001). Serotonin-induced DA release in the NAc is believed to be mediated through the activation of 5-HT receptors such as 5-HT_{1A}, 5-HT_3 and 5-HT_{2A} in the NAc (Campbell and McBride, 1995; Ichikawa and Meltzer, 2000; Lucas and Spampinato, 2000). The excitatory-life effects of 5-HT_{1A}, 5-HT_3 and 5-HT_{2A} receptors on DA release in the NAC is balanced by the inhibitory effects of 5-

HT_{2c} receptors that activate GABA neurons that project to the DRN causing a reduction in 5-HT firing cells (Tao and Auerbach, 2000; Di Matteo et al., 2001). Therefore, an imbalance between the excitatory and inhibitory 5-HT receptors might alter DA release in the NAc. As a result, it is also possible that MS and CCL might have altered the activity of 5-HT_{1A}, 5-HT_3 and 5-HT_{2A} receptors by desensitizing them and causing a reduction in 5-HT levels. This would in turn induce a decrease in [^{3}H]DA release in the NAc. It is also possible that the effects of CCL and MS might have increased the number of 5-HT_{2C} receptors causing more inhibitory activity in the NAc. As a result, the inhibitory effects of 5-HT_{2C}R would decrease 5-HT levels and reduce glutamate-stimulated [^{3}H]DA release in the NAc.

Further evidence of the 5-HT-induced DA activity in the NAc originates from the use of antidepressant (SSRIs) which increase extracellular levels of 5-HT by blocking its re-uptake into the presynaptic cell (McEwen et al., 1993; Deana et al., 2001). Serotonergic neurons within the NAc are essential for regulating dopaminergic functions; motivation, reward and various forms of stress and it is believed that these functions are mediated by the antidepressant effects of SSRIs (McEwen et al., 1993; Deanna et al., 2001). Moreover, the fast onset antidepressants act through various 5-HT receptors thereby regulating DA release in the NAc (Parsons and Justice, 1993; Campbell and McBride, 1995; Ichikawa and Meltzer, 1999; Lucas and Spampinato, 2000; Di Matteo et al., 2001). For example, reduced extracellular DA levels in FSL rats were elevated after administration of nefazondine, a potent 5-HT_{2C} antagonist (Sanchez et al., 1999; Dremencov et al., 2004; Wesolowska et al., 2006). The 5-HT_{2C} receptor antagonist induces an immediate increase in DA levels in the NAc of FSL rats (Dremencov et al., 2005). Moreover, the ability of 5-HT to induce DA release was also correlated with improvements of depressive-like behaviour in FSL rats (Dremencov et al., 2005).

4.2 Serotonin levels in the hypothalamus and PFC are altered by the effects of MS and CCL

In addition to altered [^{3}H]DA release in the NAc by MS and CCL it was further demonstrated that 5-HT levels in the hypothalamus and PFC were also altered by MS and CCL. In the hypothalamus, CCL exposure caused an overall increase in 5-HT levels whereby both NMS and MS rats displayed a significant increase in 5-HT levels than NMS and MS rats without CCL exposure. Despite not showing a statistical difference, the MS rats subjected to CCL exposure showed augmented 5-HT levels in the hypothalamus and this increase was greater than the increased 5-HT levels of NMS rats subjected to CCL, suggesting that the combination of MS and CCL is a greater stressor and hence has a greater effect in altering 5-HT levels than when MS or CCL is used as the sole stressor. On the other hand, in the PFC, NMS and MS rats exposed to CCL exhibited reduced 5-HT levels in comparison to NMS rats. Similarly, addition of CCL further reduced 5-HT levels in the PFC of MS rats compared to MS alone, as the sole stressor. Therefore, addition of CCL exaggerates the effects of MS causing alterations in 5HT levels in the hypothalamus and PFC.

The 5HT-ergic response to different stressors varies, as demonstrated by the fact that stress increases or decreases or has no effect on extracellular levels of 5-HT in different brain areas such as the PFC, hippocampus and amygdala (Kirby et al., 1995; Kirby and Lucki, 1997). Moreover, the reactivity of the 5HT-ergic system is inconsistent and varies with the type of stressor or brain area (Keeney et al., 2006). This might explain the disparate 5-HT levels found in the hypothalamus and PFC, further providing evidence of the reactivity of the 5HT-ergic system in different brain regions.

Altered 5-HT levels in the rat brain have been reported in other animal models of depression and other stress paradigms (Zangen et al., 1997; Rentesi et al., 2010). In a genetic rat model of depression, FSL, 5-HT and its metabolites (5-HIAA) were significantly higher in the hypothalamus, NAc and PFC compared to control rats (Zangen et al., 1997). Moreover, it was reported that the increased 5-HT levels

correlated with high immobility in the FST, which is indicative of depressive-like behaviours.

Furthermore, different forms of stress (swimming, cat exposure, tail pinch) increased 5-HT levels (20-65%) above baseline levels in the PFC (Rueter and Jacobs, 1996). Increased 5-HT levels in the hypothalamus and increased anxiety-like behaviour in the OFT were also reported after MS (Rentesi et al., 2010). Moreover, MS rats displayed elevated ACTH and CORT levels consistent with the stressful effects of MS (Rentesi et al., 2010). On the other hand, decreased 5-HT levels were reported in the hippocampus, PFC and amygdala of rats subjected to MS compared to NMS rats (Matthew et al., 2001; Lee et al., 2007). The discrepancies might be due to duration of the MS paradigm, whereby MS rats in the study conducted by Rentesi et al. (2010) were separated for 24 hours from the dams for PND 9. This is in contrast to the 3 and 6 hour daily MS procedure performed by Matthew et al. (2001) and Lee et al. (2007) over a two week period.

These studies further indicate that the 5HT-ergic system is highly susceptible to various forms of stressors, further providing evidence of the effects of MS and CCL exposure in altering 5-HT levels in the hypothalamus and PFC. Reduced 5-HT levels attenuate the stress response whereas increased 5-HT levels assist animals in responding to stress (Mo et al., 2008). According to Mo et al. (2008) restraint stress increases 5-HT levels in the amygdala by activating CRF which is secreted by the PVN of the hypothalamus in response to stress. Therefore, it is plausible that the elevated 5HT-levels in the hypothalamus of MS and CCL exposed animals might have activated CRF secretion in order to cope with the effects of the combined stress of MS and CCL.

The altered 5-HT levels in the hypothalamus and PFC might also suggest abnormal activity of the DRN. The DRN contains about 50% 5HT-ergic cells (Azmitta and Segel, 1978; Jacobs and Azmitta, 1992). The DRN innervates the forebrain, NAc and hypothalamus and these are important brain regions regulating mood and stress responses (Di Matteo et al., 2001; Molliver, 1989; Deyron et al., 1998; Mantz et al., 1990). The 5HT-ergic neurons from the DRN innervate the SCN thereby conveying information about locomotor activity (Bailey and Silver,

2014). Furthermore, the SCN plays an important role in regulating circadian rhythms of many biological processes such as melatonin secretion (Klein and Moore; Campbell and Dawson, 1990; Redlin et al., 2001). For example, melatonin production is regulated by the SCN and under the normal L/D cycle, the light period activates the SCN and suppresses melatonin secretion (Klein, 1993; Karolczak et al., 2005; Escobar et al., 2011). Melatonin production is also regulated by stress exposure (Reiter et al., 2000). Therefore, it is proposed that MS and CCL exposure might have impaired SCN activity thereby inhibiting melatonin production and increasing the risk of developing depression. This claim is in agreement with the claim that melatonin has antidepressant-like actions whereas deficiencies in melatonin production are implicated in depressive disorders (Beck-Frils et al., 1985; Soutre et al., 1989; Rogers et al., 2003; Crasson et al., 2004). Furthermore, agomelatine an analogue of melatonin has antidepressant properties and high affinity for 5-HT_{2C} receptors (Cussac et al., 2002; Millan, 2003). It is believed that the therapeutic effects of agomelatine are mediated through 5-HT_{2C} receptors (Cussac et al., 2002; Millan, 2003). These findings are also in agreement with the studies demonstrating that inhibition of 5-HT_{2C} receptors in the NAc elevates DA release leading to an improvement in depressive-like behaviours (Campbell and McBride, 1995; Ichikawa and Meltzer, 1999; Di Matteo et al., 2001; Dremencov et al., 2005).

Moreover, MT1 and 5-HT_{2C} receptors are expressed in the SCN and in brain regions implicated in the pathophysiology of depression (San and Arranz, 2008). Most importantly, the 5-HT_{2C} receptor is the only 5-HT receptor that exhibits a CR in expression (Holmes et al., 1997). Furthermore, a 5-HT_{2C} receptor agonist has a similar effect as a light pulse and inhibits melatonin production. Conversely, a 5-HT_{2C} receptor antagonist prevents the inhibitory effect of light on melatonin synthesis (Kennaway and Emsley, 2006). Thus if chronic stress increases 5-HT_{2C} receptor expression and 5-HT_{2C} agonists have an effect that is similar to that of light pulses, it is possible that the effects of CCL and MS might have altered 5-HT_{2C} receptors in the hypothalamus and PFC. Therefore, increased 5-HT_{2C}R might also explain the reduced 5-HT levels found in the PFC. However, increased 5-HT_{2C}R does not explain the increased 5-HT levels in the hypothalamus. Therefore, it can be suggested that increased 5-HT levels in the hypothalamus

might be due to a compensatory response by the SCN in order to minimise the effects of CCL stress.

Therefore, CCL might have also increased 5-HT_{2C}R leading to the suppression of melatonin secretion implying that CCL is a stressor (Millan et al., 2003; Papp et al., 2003; Bourin et al., 2004). These results also suggest with suppressed melatonin secretion and increased 5-HT_{2C}R results in increased vulnerability to anhedonic and depressive like behaviours. Consistent with this suggestion, Moreau et al. (1996), showed that stressed rats that had an increased anhedonia index, had improved anhedonic like behaviours after chronic antidepressant treatment which resulted in desensitized or down-regulated 5-HT_{2C} receptors, further suggesting that enhanced 5-HT_{2C}R activity is implicated in depression. It is possible that the combination of MS and CCL might have altered 5-HT levels in the hypothalamus and PFC by impairing DRN, SCN and 5-HT_{2C}R activity within these regions. Future studies are needed to validate the role of these brain regions in mediating light-induced 5-HT activity and test whether abnormalities within these regions plays a role in depressive behaviours. Several studies have reported anhedonic, anxiety and depressive-like behaviours after continuous light exposure suggesting that CCL acts as a chronic stressor (Schweizer et al., 2009; Martynhak et al., 2011; Tapio-Osorio et al., 2013).

4.3 MOR-1 protein levels in the NAc are resistant to the effects of MS and CCL whereas OXR-1 and OXR-2 protein levels in the PFC are altered by MS and CCL exposure

MOR-1 protein levels were not altered by the effects of MS or CCL in the NAc core and shell. Moreover, there were no significant differences in MOR-1 protein levels when NAc core and shell were averaged to obtain a representation of the function of the NAc as a whole. In contrast to the present findings, rats subjected to MS were found to have decreased MOR-1 levels in the NAc which were restored by exposure to CCL (Dimatelis et al., 2012). These findings are contradictory since both studies were performed using the same MS and CCL paradigm. Discrepancies in these studies might be due to genetic variations between

animals, meaning the animals used in this study were more resistant to the effects of MS and CCL compared to the animals used in the previous study. Furthermore, the fact that SD rats are an outbred strain supports these claims (Wu and Wang, 2010).

Similar observations have been reported in other opioid receptors, such as opioid receptor-like (ORL1) and KOR whereby there were no significant differences reported in opioid receptor-like (ORL1) and KOR between MS rats and control rats (Ploj and Nylander, 2003). Instead, MS increased DOR density in the basomedial amygdala compared to control rats (Ploj and Nylander, 2003). These results provide further evidence of the complexity of the opioid system and suggest that the different opioid receptor subtypes have opposing reactions to stressors (Lutz and Keiffer, 2013).

Nevertheless, several studies suggest that MOR is involved in stress responsivity (Unterwald et al., 1992; Nikulina et al., 1999). Social defeat stress increased MOR mRNA levels in the VTA and chronic cocaine exposure upregulated MOR density in the NAc (Unterwald et al., 1992; Nikulina et al., 1999). Furthermore, MOR has also been shown to induce anxiolytic and antidepressant properties (Filliol et al., 2000; Ide et al., 2010; Komatsu et al., 2011). For example, Filliol et al. (2000) reported less anxious and reduced depressive-like behaviours in MO knock-out mice compared to the wild type mice. It is evident that MOR is implicated in depression; however the present findings in the MOR protein levels suggest that more research is required to elucidate the function of MOR in the pathophysiology of depression. Moreover, future research should also determine the combined effects of MS and CCL on DOR and KOR activities in the hope to gain more insight into the role of the opioid system in depression. The combined effects of MS and CCL are more likely to reveal the function of DOR and KOR in depression than when either MS or CCL is used as a sole stressor.

MS and CCL exposure had an overall effect on OXR-1 protein levels in the PFC. Specifically, OXR-1 protein levels of MS rats were significantly increased compared to NMS and MS CCL rats. Similar results were found when OXR-1 protein levels were normalised. Whereby, MS rats had significantly increased

OXR-1 protein levels when compared to NMS rats further providing evidence of the effect of MS exposure on OXR-1 protein levels. On the other hand, OXR-2 proteins levels in the PFC were not altered (when only the pixel total (%) was analysed) by the effects of MS or CCL. Instead, a trend towards an increase in OXR-2 protein levels was observed was observed after MS and CCL exposure. When the OXR-2 protein levels were normalised an interaction between MS and CCL effect was reported whereby MS rats had increased OXR-2 protein levels compared to NMS and MS CCL rats. Furthermore, NMS CCL rats displayed a trend towards increase in OXR-2 protein levels when compared to NMS rats. These findings suggest that OXR-1 and OXR-2 protein levels are significantly influenced by the effects of MS and CCL exposure.

Similarly, other studies have demonstrated changes in OXR-1 or in both OXR-1 and OXR-2 receptors following various forms of stress (Karteris et al., 2005; Feng et al., 2007; Scott et al., 2011; Arendt et al., 2013). For example, Feng et al. (2007) found increased OXR-1 protein levels in the PFC and increased orexin A peptide in the hypothalamus of rats subjected to MS. Furthermore, increased OXR-1 and orexin A peptide levels were proposed to have caused a reduction in total sleep as recorded by an electroencephalogram (EEG) during the light period (corresponds to night time in humans). Therefore, the increased OXR-1 protein levels might lead to sleep disturbances and circadian abnormalities, phenotypes that are well characterised in depression due to an altered orexinergic system (Peyron et al., 2000; Salomon et al., 2003; Sakurai et al., 2010; Cao and Guilleminault, 2011).

Furthermore, increased OXR-1 mRNA expression in the amygdala was positively correlated with increased depressive-like behaviours in the FST (Arendt et al., 2013). The role of OXR-1 in depression is further supported by the findings that OXR-1 KO mice exhibit decreased immobility time in the FST, indicating reduced depressive-like behaviours (Scott et al., 2011). Moreover, wild type mice treated with an OXR-1 antagonist also display reduced immobility time in the FST, indicating antidepressant properties (Scott et al., 2011). Overall, these studies provide substantial evidence of the role of OXR-1 in depression and also lend

support to the increased OXR-1 protein levels after MS, suggesting that OXR-1 is associated with depressed moods.

It has further been postulated that activation of orexin neurons that project to many brain areas expressing OXR-1 during stressful events might induce a negative affective state that can exacerbate depressive-like behaviours (Scott et al., 2011). Moreover, further involvement of OXR-1 in depression comes from a study by Rainero et al. (2011) who reported that allelic (specifically the A allele) and genotypic frequencies of the polymorphism (rs2271933 G>A) in the OXR-1 gene were associated with the increased risk of developing MDD. Collectively, these findings highlight the importance of OXR-1 in modulating depressed moods and also suggest that drugs acting on OXR-1 might be useful in treating depression.

Increases in both OXR-1 and OXR-2 protein levels following stress exposure have also been by Karteris et al. (2005). In this study, OXR-1 and OXR-2 protein levels were significantly increased by a 24 hour food deprivation, another stress paradigm (Karteris et al., 2005). Therefore, the increased OXR-1 and OXR-2 (trend and increased OXR-2 protein levels after normalisation) protein levels in the PFC and in the hypothalamus as indicated by Karteris et al. (2005) suggest that these receptors are implicated in the regulation of the HPA axis, an important system mediating stress responses (Lupien et al., 2009). Both the PFC and the hypothalamus control the activity of the HPA axis and disturbances to these brain regions (i.e. altered HPA axis activity) has been associated with the risk of developing depression (Heim et al., 2000; Lupien et al., 2000; Geoffroy et al., 2006; Albers et al., 2008; Lupien et al., 2009). Thus the increased OXR-1 and OXR-2 protein levels in the PFC as a result of MS and CCL exposure indicate that orexin receptors are involved in the stress regulation. It is possible that the altered orexin receptors might have impaired the HPA axis activity leading to depression. These results are in accordance with the findings reported by various authors that have also implicated the orexin receptors in the pathophysiology of depression (Karteris et al., 2005; Feng et al., 2007; Scott et al., 2011; Nollet et al., 2011; Arendt et al., 2013).

CHAPTER 5: CONCLUSION

This study demonstrated that MS altered [^{3}H]DA release in the NAc. In addition, CCL exacerbated the reduction of [^{3}H]DA release in the NAc. Furthermore, 5-HT levels in the hypothalamus and PFC were altered by the effects of MS and CCL. These findings add to the existing literature on the long-term effect of MS during early brain development and confirm that early life experiences can lead to changes in brain development that can influence brain function and affect how the brain responds to additional stressors in adulthood (Hout et al., 2001; Vasquez et al., 2005; Aisa et al., 2007; Marais et al., 2008; Reus et al., 2011).

Glutamate-stimulated [^{3}H]DA release in the NAc was significantly decreased in MS rats compared to the NMS rats**.** It was also revealed that addition of CCL in adulthood in MS rats aggravated the reduction of [^{3}H]DA release in the NAc shell, however no statistical significance was reached. On the other hand, CCL exposure significantly altered 5-HT levels in the hypothalamus and PFC both in MS and NMS rats. It is important to note that addition of CCL exaggerated the effects of MS, whereby MS and CCL combined caused a decrease in 5-HT levels in the PFC. Therefore, addition of chronic light exposure during adulthood provides more knowledge about the neurobiological mechanisms of MS-induced depression. These findings also suggest that the combination of MS and CCL is better than when either MS or CCL as used as a sole stressor.

Despite these significant differences in DA release and 5-HT levels, the precise mechanisms leading to the decreased glutamate-stimulated [^{3}H]DA release in the NAc and altered 5-HT levels in the hypothalamus and PFC are not fully understood. Thus, research is still needed to elucidate the role of DA and 5-HT in depression. Further work that deciphers the role of MS in reducing [^{3}H]DA release in the NAc might assist in providing more detailed insights into the systems that mediate DA release such as the glutamatergic and 5HT-ergic systems. Emphasis should be on the interaction between glutamate group II metabotropic receptors (in particular mGluR2/3) and the DA system in regulating DA releases Moreover, it is important to investigate how CCL influences the 5HT-ergic system and to test

whether this influence changes DA release in the NAc. Future work can clarify whether an imbalance in 5-HT levels causes a reduction in DA release in the NAc.

Similarly, future studies should also investigate if additional exposure to CCL in MS rats alters 5-HT receptors, particularly in the hypothalamus and PFC in order to determine if this has a causal effect on the altered 5-HT levels in the hypothalamus and PFC. Future research should also measure DRN activity, since this is the brain region where most 5-HT cells are found. Particularly, future research should investigate whether projection of these cells from DRN to the hypothalamus and PFC causes alterations to the 5-HT levels in the hypothalamus and PFC. It is also proposed that investigation of the 5-HT_{2C}R levels in the hypothalamus and PFC following MS and CCL exposure might assist in explaining the altered 5-HT levels in these brain regions. Especially that 5-HT_{2C}R has been implicated in depression (Campbell and McBride, 1995; Ichikawa and Meltzer, 1999; Di Matteo et al., 2001; Cussac et al., 2002; Millan, 2003; Dremencov et al., 2005).

On the other hand, MS and CCL did not alter MOR-1 levels in the NAc. Due to the conflicting findings reported on the opioid system, in particular MOR activity in depression. Future work should investigate the role of MOR activity in the pathophysiology of depression and also determine the combined effects of MS and CCL on DOR and KOR activities in the hope to gain more insight into the role of the opioid system in depression.

This study has also added to the limited literature on describing the role of orexinergic system in depression. The increased OXR-1 protein levels in depression are agreement with several studies that have also documented increased OXR-1 protein levels as well as altered orexinergic system in depression (Karteris et al., 2005; Feng et al., 2007; Scott et al., 2011; Arendt et al., 2013). Similarly, the increased trend towards significance in OXR-2 protein levels (when the pixel total % was analysed) and the significant increase in OXR-2 protein levels after the OXR-2 was normalised against p38 provide evidence of the role of OXR-2 in depression. Therefore, OXR-1 and OXR-2 protein levels are significantly altered by the effects of MS and CCL exposure. Of importance to note is that the additional exposure to CCL exercabated the effects of MS.

This study has demonstrated that CCL acts as a stressor, despite not measuring HPA activity in response to CCL exposure. Various studies have reported a dysregulated HPA axis after light exposure, thus providing support of CCL involvement in the HPA axis activity (Blom et al., 1995; Fischeman et al., 1988; Mohawk et al., 2007). Thus highlighting the importance of using the CCL paradigm in studying depression as well as studying the pathophysiology of bipolar disorder, as previously proposed by other authors (Canal-corretger et al., 2001; Flaisher-Grinberg et al., 2009; Martynhak et al., 2011).

In conclusion, this study has provided evidence that CCL exaggerates the effects of MS and that addition of CCL created a more robust model of MS.

APPENDICES

Appendix A: Ethics approval letter

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences
Animal Ethics Committee
Room E53-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 404 7682 • **Facsimile** [021] 406 6411
e-mail: nosi.tsama@uct.ac.za

27 June 2013

AEC REF NO: 012/057

Prof V Russell
Human Biology
Falmouth Building

Dear Prof Russell

Project Title: Molecular mechanisms involved in the anti-depressant effect of light in the treatment of maternal separation-induced depression in rats.

Thank you for submitting your amendment to the Faculty of Health Sciences Animal Ethics Committee for review.

It is a pleasure to inform you that the FHS AEC has authorised the use of 2 additional experimental rats for the above mentioned study.

A form for amendments (Version August 2013) is also available at Website address:
http://www.health.uct.ac.za/research/animalethics/forms/
Please submit a yearly progress reports to ethics office. This is a requirement for ongoing approval of studies.
Notification of study closure is also a requirement.
Ethics authorisation letters and copy of the application to be submitted to the Animal Unit when commencing the study for the release of animals

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROF GRAHAM LOUW
CHAIR, HSF AEC

Appendix B: In vitro superfusion

B1: Krebs buffer reagents

118 mM Nacl,
4.7 mM KCl,
1.0 mM NaH_2PO_4
1.2 mM $MgCl_2$
1.3 mM $Cacl_2$
2.5 mM $NaHCO_3$
11 mM glucose

Add 0.04 mM EDTA

Make up to 1 litre

B2: Quench standard curve

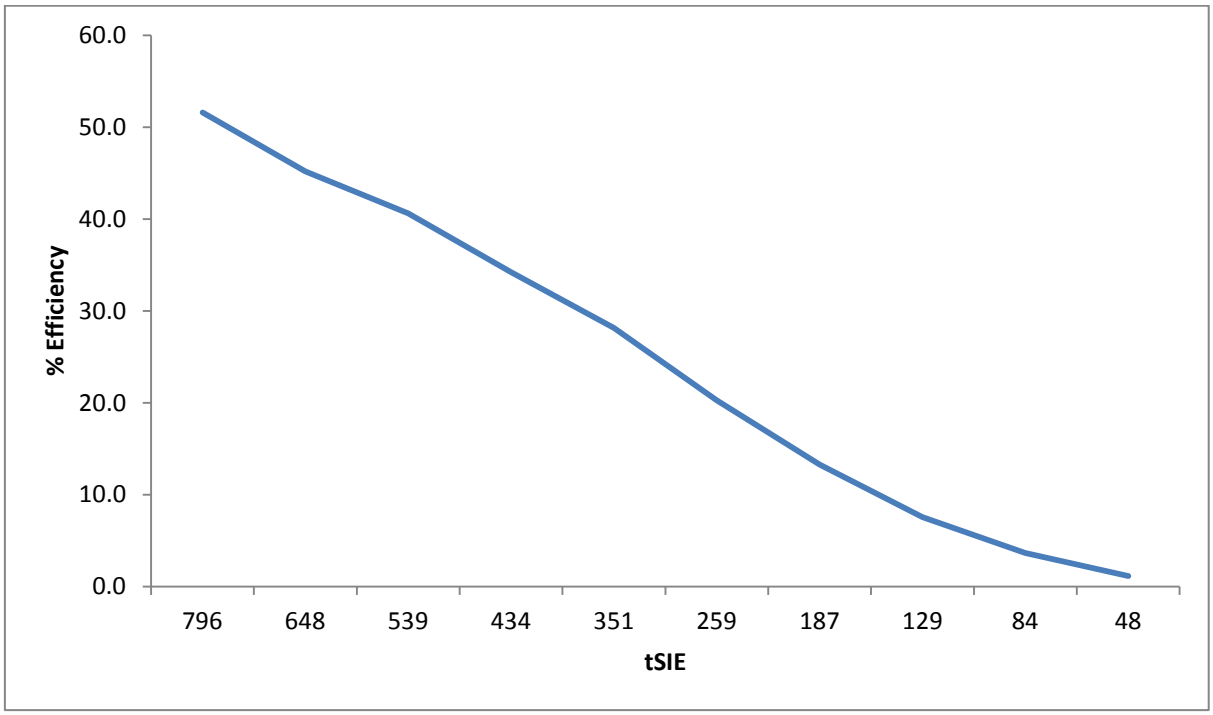
Standard	CPM	DPM	% Efficiency	tSIE
1	83027	160993	51,6	796
2	73222	162134	45,2	648
3	65451	161099	40,6	539
4	55163	161212	34,2	434
5	45412	161428	28,1	351
6	32517	160281	20,3	259
7	21578	162650	13,3	187
8	12320	162916	7,6	129
9	5914	162406	3,6	84
10	1921	167744	1,1	48

CPM= Counts per minute

DPM= Disintegrations per minute

% Efficiency= (CPM/DPM) * 100

tSIE= Transformed index of the external standard spectrum



The tSIE of the NAC core and shell samples was within the range of 200-400 tSIE values.

Appendix C: ELISA

C1: RIPA Buffer

REAGENT	STOCK CONCENTRATION	FINAL CONCENTRATION	FOR 50 ml
NaCl	5 M	150mM	1.5 ml
Triton X-100	100%	1%	500 ul
SDS	10%	0.1%	500 ul
Tris(pH 7.5)	1 M	20 mM	1 ml
Deoxycholate	Powder	1%	0.5 g
dH_2O	-	-	46.5 ml

Add 2 µg of Protein Inhibitor cocktail per 1 ml of RIPA buffer

1.5 M Tris-HCl, pH 8.8

Tris base mol. Wt = 121.14

18.17 g Tris base in 70 ml dH_2O
Adjust pH with 10 N HCl
Bring total volume to 100 ml
Store at 4°C

0.5 M Tris-HCl, pH 6.8

Tris base mol. Wt = 121.14

6 g Tris base in 70 ml dH_2O
Adjust pH with 10 N HCl
Bring total volume to 100 ml
Store at 4°C

10 % SDS

100g SDS salt
Make up to 1 litre with dH_2O

C2: Reagents

Symbol	Component
MTP	**Microtiter Plate** Break apart strips. Coated with anti-rabbit antiserum (goat).
ANTISERUM	**Serotonin Antiserum** Blue colored. Ready to use. Contains: Antiserum (rabbit), phosphate buffer, < 0.1 % NaN_3.
BIOTIN	**Serotonin Biotin** Yellow Colored. Ready to use. Contains: < 0.1 % NaN_3.
ENZCONJ CONC	**Enzyme Conjugate**, Concentrate (100x) Contains: streptavidin alkaline phosphatase, Tris buffer, HCl, < 0.1 % NaN_3.
CAL A-G	**Standard A-G** 0; 0.08; 0.24; 0.73; 2.2; 6.6; 19.8 ng/mL 0; 0.45; 1.4; 4.1; 12.5; 37.4; 112.3 nmol/L Ready to use. Contains: Serotonin (acylated), phosphate buffer, < 0.1 % NaN_3.
CONTROL 1+2 LYO	**Control 1+2**, lyophilized Contains: Human serum, < 0.1 % NaN_3. Concentrations / acceptable ranges see QC Certificate.
ACYLREAG	**Acylation Reagent** Acetic Acid Anhydride, acetone. Ready to use.
ASSAYBUF CONC	**Assay Buffer** Concentrate (10x) Contains: phosphate buffer, BSA, < 1 % NaN_3.
WASHBUF CONC	**Wash Buffer** Concentrate (20x) Contains: phosphate buffer, Tween, < 0.1 % Thimerosal.
PNPP SUBS	**PNPP Substrate Solution** Ready to use. Contains: p-nitrophenyl phosphate (PNPP).
PNPP STOP	**PNPP Stop Solution** Ready to use. Contains: 1 M NaOH

C3: 5-HT quality control certificate for the hypothalamus samples

Quality Control Certificate

Serotonin ELISA

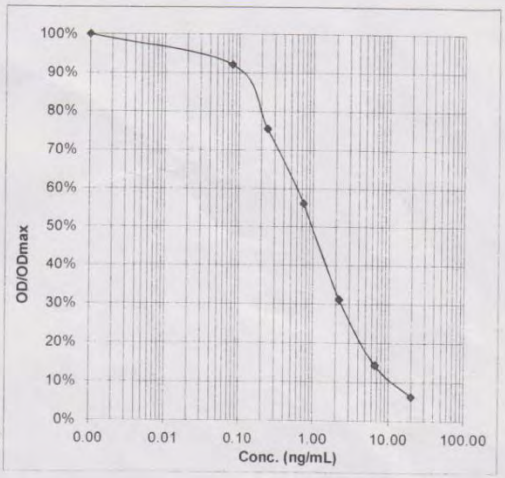
IBL INTERNATIONAL

REF RE59121 | LOT ESE119 | 140331 (YY MM DD) | IVD CE

Final Release Results:

	Concentration ng/mL	manual: Measured value OD	manual: Range OD	manual: OD/ODmax	automated: Measured value OD
CAL A	0.00	2.581	> 1.000	100%	2.998
CAL B	0.08	2.378	-	92%	2.885
CAL C	0.24	1.948	-	75%	2.784
CAL D	0.73	1.449	-	56%	2.210
CAL E	2.2	0.807	-	31%	1.410
CAL F	6.6	0.372	-	14%	0.713
CAL G	19.8	0.162	< 0.500	6%	0.329

	Concentration found ng/mL	Acceptable range: lower limit ng/mL	target ng/mL	upper limit ng/mL
CONTROL 1	79	54	91	127
CONTROL 2	241	183	261	340



Kit Composition:

REF		LOT	
KESE651	LYO CONTROL 1	W925	2015-05
KESE661	LYO CONTROL 2	W926	2015-05
KEZZ811	MTP	W725	2016-02
KESE521	ANTISERUM	W717-A	2014-03
KESE531	BIOTIN	W928	2014-06
KESE541	CONC ENZCONJ	W939	2014-12
KESE611	CAL A-G	W775-A	2014-03

REF		LOT	
KESE761	ACYLREAG	W929	2014-03
KEZZ681	CONC ASSAYBUF	W924	2014-12
KEZZ701	CONC WASHBUF	W918	2015-04
KEZZ961	PNPP SUBS	W940	2015-08
KEZZ951	PNPP STOP	W777	2016-03

Valid edition of the instructions for use: V2012-12

This product has been tested successfully by the Quality Control Department and was released for sale according to the existing specifications.

Verified by: Dr. Spangenberg

Accepted

Validation date: 2013-05-30

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C4: 5-HT quality control certificate for PFC samples

Quality Control Certificate

PFC

IBL INTERNATIONAL

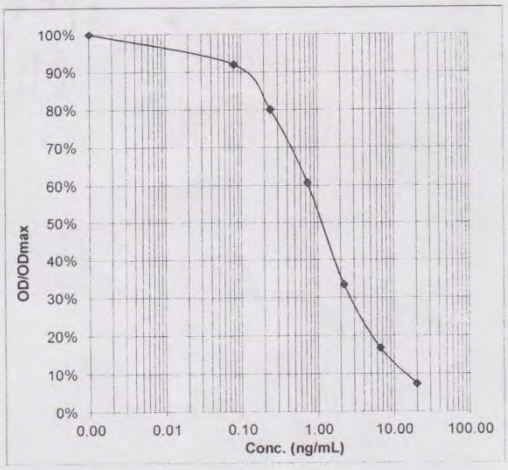
Serotonin ELISA

REF RE59121 | LOT ESE121A | 140630 (YY MM DD) | IVD CE

Final Release Results:

	Concentration ng/mL	manual: Measured value OD	manual: Range OD	manual: OD/ODmax	automated: Measured value OD
CAL A	0.00	1.858	> 1.000	100%	2.294
CAL B	0.08	1.709	-	92%	2.204
CAL C	0.24	1.488	-	80%	1.873
CAL D	0.73	1.128	-	61%	1.607
CAL E	2.2	0.621	-	33%	1.152
CAL F	6.6	0.312	-	17%	0.587
CAL G	19.8	0.136	< 0.500	7%	0.258

	Concentration found ng/mL	Acceptable range: lower limit ng/mL	target ng/mL	upper limit ng/mL
CONTROL 1	123	77	128	179
CONTROL 2	444	319	455	592



Kit Composition:

REF		LOT	
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KESE661	LYO CONTROL 2	V498	2015-10
KEZZ811	MTP	V298	2016-07
KESE521	ANTISERUM	V267	2014-06
KESE531	BIOTIN	W928-A	2014-09
KESE541	CONC ENZCONJ	V415	2015-04
KESE611	CAL A-G	W775-2B	2014-06

REF		LOT	
KESE761	ACYLREAG	V307	2014-06
KEZZ681	CONC ASSAYBUF	V308	2015-03
KEZZ701	CONC WASHBUF	V283	2015-07
KEZZ961	PNPP SUBS	V266	2015-08
KEZZ951	PNPP STOP	W777	2016-03

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Appendix D: Western blot

D1: BCA protein assay procedure

1. Thaw the following BSA protein standards on ice:
   A: 2000 µg/ml
   C: 1000 µg/ml
   E: 500 µg/ml
   G: 125 µg/ml
   I: 0 µg/ml

2. Dilute sample proteins 1/6 in RIPA Extraction Buffer as follows:

   10ul of protein (already in Eppie) + 50ul of RIPA (**without complete protease inhibitors**).

3. Calculate how much Working Reagent (WR) to make up (you need 200 µl/well).

   e.g. (2 samples + 5 stds) x 2 (for duplicate) +1 (for pipetting error) = 15 x 200µl = 3ml

   To make up WR: 50(A):1(B)
   3ml(A) + 60µl(B)

   Add A first, followed by B. Mix until WR is green in colour

4. Pipette 25µl of stds and samples in duplicate into wells of flat-bottom 96 well plate (record order of stds and samples in your plate)

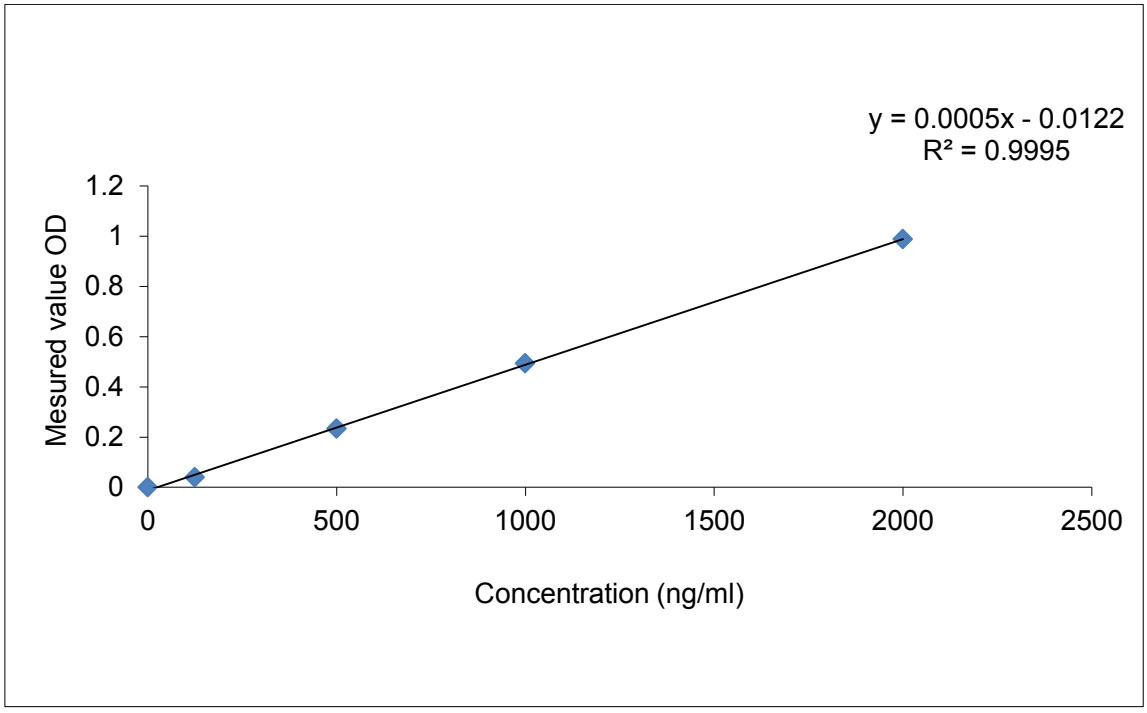
5. Add 200µl of WR to each well – Sample will now turn purple: the darker the sample, the greater the [protein]

6. Shake briefly so that WR and samples mix (don't spill)

7. Cover plate with Parafilm and incubate at 37°C for 30mins.

D2: Example of a BCA standard curve

Standards (ng/ml)	OD1	OD2	OD Ave	OD - blank
2000	1,224	1,224	1,224	0,9885
1000	0,737	0,72	0,7285	0,493
500	0,468	0,469	0,4685	0,233
125	0,275	0,274	0,2745	0,039
0	0,241	0,23	0,2355	0



D3: SDS-PAGE recipe for 4 gels (large proteins)

REAGENT	12 % SEPARATING GEL	5% STACKING GEL
dH2O	4.9 ml	4.8 ml
Acrylamide:bis (30%)	6 ml	1.060 ml
1.5M Tris buffer pH 8.8	3.8 ml	-
0.5M Tris buffer pH 6.8	-	2 ml
10% SDS	150 µl	80 µl
10% Ammonium persulphate *	150 µl	100 µl
Temed (add just before pouring!)*	15 µl	20 µl

***Add last, as this will start setting the gel**

D4: SDS-PAGE recipe for 4 gels (small proteins)

Reagent	18 % Separating gel	5 % Stacking gel
Ethylene glycol	9.6 ml	_
3M Tris-HCl buffer ,p H 8.45	8 ml	4 ml
Acrylamide/bisacrylamide (40%)	19 ml	2 ml
Deionized water	-	10 ml
40% APS	32 µl	16 µl
TEMED (100%)*	48 µl	64 µl

***Add last, as this will start setting the gel**

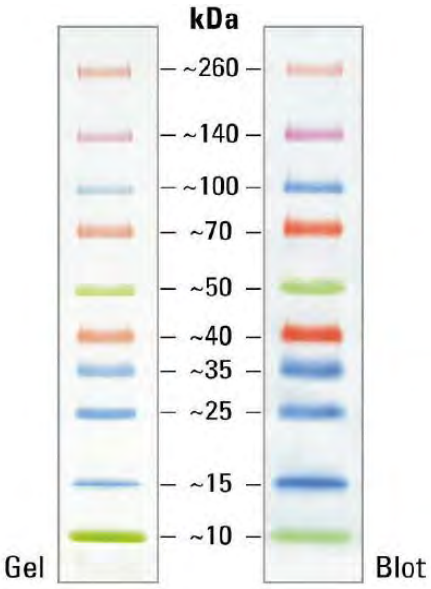
10% Ammonium Persulphate (APS)

Always prepare fresh

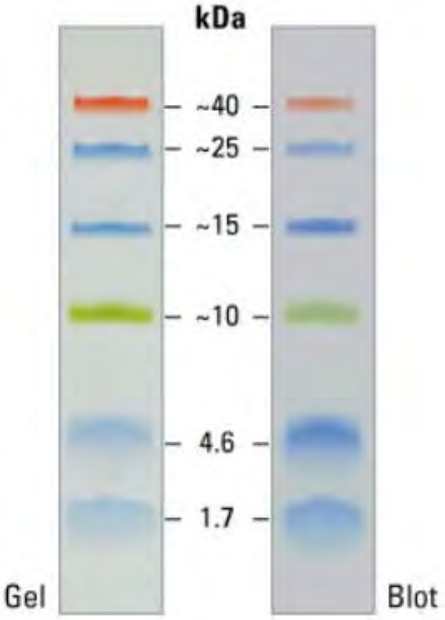
For 1 ml:

0.1 g ammonium persulphate
1 ml dH_2O

D5: Thermo Scientific Spectra Multicolor Protein Ladder



(Broad range)



(Low Range)

D6: Western Blot Buffers

Stripping buffer

0.2M NaOH

Weigh 8 g of NaOH pellets into a flask

Add in 1 L of distilled H_20 (stir until the pellets are completely dissolved)

Tank buffer (1L)

3 g Tris – mol. WT = 121.14
14.4 g Glycine
10 ml 10% SDS
Make up to 1 litre with dH_2O

4X Transfer Buffer

43 g Tris base
109.45 g Glycine
Make up to 2 litres with dH_2O

1X Transfer Buffer

Make long before use and store at 4°C

250 ml 4X transfer Buffer
200 ml isopropanol
Make up to 1 litre with dH_2O

1X PBS-Tween (PBS-T) 2L

Add:
100 ml 10X PBS
900 ml dH_2O
1 ml Tween 20

Stir for 20 minutes

10X PBS:

80g NaCl
2g KCl
14.4g Na2HPO4
2.4g KH2PO4

Appendix E: List of equipments

Sonicator: MSE Soniprep 150,MSE Scientific Instruments, England

Centrifudge: Prism Refrigerated Microcentrifuge, Labnet International Inc, USA

Vortex machine: Vortex-Genie 2, Scientific Industries, USA

Spectrophotometer, Multiskan FC Thermo Fisher Scientific, Shanghai

Water bath: Julabo, Paratherm, 5100 electronic waterbath

Perfusion pump: Watson Marlow 2055, UK

Beta counter: Packard 1900 CATRI-CARB liquid scintillation analyser

Gel and transfer apparatus: Bio-rad Power Pac universal, Bio-Rad Laboratories Inc. USA

Orbital shaker: Labnet vortemp 56, Labnet International , Inc.USA

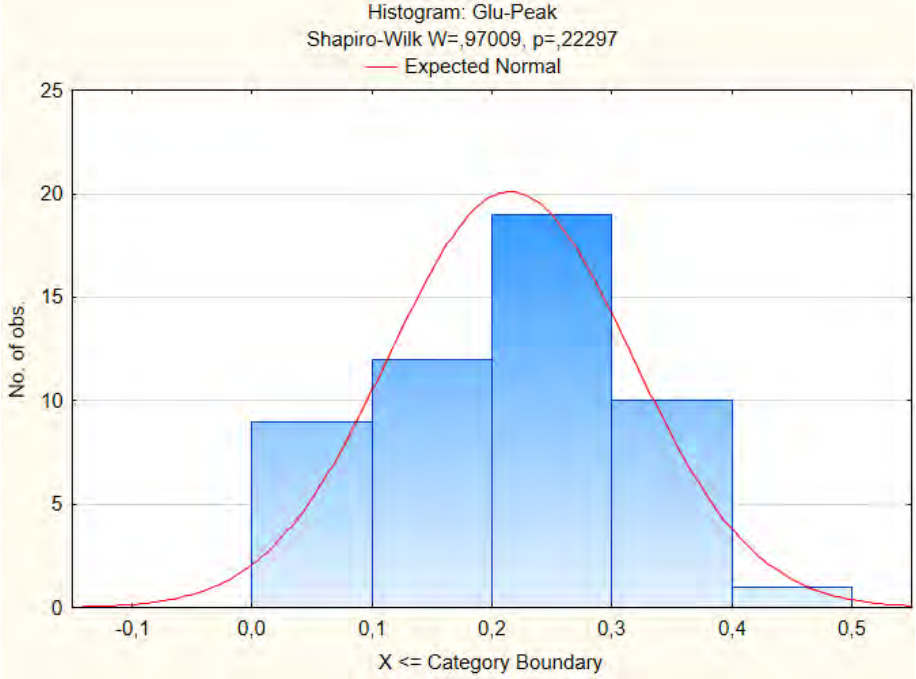
Appendix H: Statistical Data

Appendix H1: In vitro Superfusion

Glutamate-stimulated [^{3}H]DA release in the NAc core

	Glutamate-stimulated [^{3}H]DA release in the NAc core			
	Stress	Treatment	Stress x Treatment	Glu-Peak
1	nMS	nL	nMSnL	0,30295
2	nMS	nL	nMSnL	0,2221
3	nMS	nL	nMSnL	0,2083
4	nMS	nL	nMSnL	0,28685
5	nMS	nL	nMSnL	0,4279
6	nMS	nL	nMSnL	0,3164
7	nMS	nL	nMSnL	0,04355
8	nMS	nL	nMSnL	0,2667
9	nMS	nL	nMSnL	0,2136
10	nMS	nL	nMSnL	0,2006
11	nMS	nL	nMSnL	0,22755
12	nMS	nL	nMSnL	0,3031
13	nMS	nL	nMSnL	0,393
14	MS	L	MSL	0,0664
15	MS	L	MSL	0,2095
16	MS	L	MSL	0,15295
17	MS	L	MSL	0,09865
18	MS	L	MSL	0,359
19	MS	L	MSL	0,16615
20	MS	L	MSL	0,36865
21	MS	L	MSL	0,2544
22	MS	L	MSL	0,1934
23	MS	L	MSL	0,16995

	Glutamate-stimulated [^{3}H]DA release in the NAc core			
	Stress	Treatment	Stress x Treatment	Glu-Peak
24	MS	L	MSL	0,0781
25	MS	L	MSL	0,13515
26	MS	L	MSL	0,3948
27	nMS	L	nMSL	0,2854
28	nMS	L	nMSL	0,1996
29	nMS	L	nMSL	0,158055
30	nMS	L	nMSL	0,0358
31	nMS	L	nMSL	0,0689
32	nMS	L	nMSL	0,3842
33	nMS	L	nMSL	0,20585
34	nMS	L	nMSL	0,2183
35	nMS	L	nMSL	0,27135
36	nMS	L	nMSL	0,21625
37	nMS	L	nMSL	0,19695
38	MS	nL	MSnL	0,2247
39	MS	nL	MSnL	0,05285
40	MS	nL	MSnL	0,2104
41	MS	nL	MSnL	0,0969
42	MS	nL	MSnL	0,153
43	MS	nL	MSnL	0,1701
44	MS	nL	MSnL	0,39465
45	MS	nL	MSnL	0,0837
46	MS	nL	MSnL	0,23795
47	MS	nL	MSnL	0,2107
48	MS	nL	MSnL	0,12915
49	MS	nL	MSnL	0,3411
50	MS	nL	MSnL	0,25915
51	MS	nL	MSnL	0,125



Breakdown Table of Descriptive Statistics (Statistica Superfusion 31 October 2013)
N=51 (No missing data in dep. var. list)

Stress	Treatment	Glu-Peak Means	Glu-Peak N	Glu-Peak Std.Dev.	Glu-Peak Std.Err.	Glu-Peak Q25	Glu-Peak Median	Glu-Peak Q75
nMS	nL	0,262508	13	0,096238	0,026692	0,213600	0,266700	0,303100
nMS	L	0,203696	11	0,096288	0,029032	0,158055	0,205850	0,271350
MS	nL	0,192096	14	0,096974	0,025917	0,125000	0,190250	0,237950
MS	L	0,203623	13	0,110318	0,030597	0,135150	0,169950	0,254400
All Groups		0,215484	51	0,101184	0,014169	0,152950	0,210400	0,285400

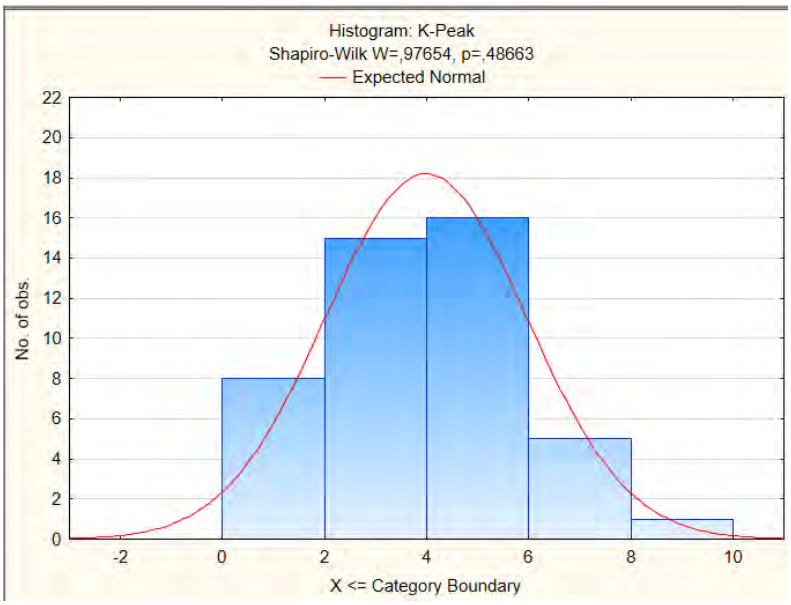
Univariate Tests of Significance for Glu-Peak (Statistica Superfusion 31 October 2013)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	2,349619	1	2,349619	233,8929	0,000000
Stress	0,015712	1	0,015712	1,5641	0,217258
Treatment	0,007071	1	0,007071	0,7039	0,405714
Stress*Treatment	0,015648	1	0,015648	1,5576	0,218194
Error	0,472148	47	0,010046		

Potassium-stimulated [^{3}H]DA release in the NAc core

	Potassium-stimulated [^{3}H]DA release in the NAc core			
	Stress	Treatment	Stress x Treatment	K-Peak
1	nMS	nL	nMSnL	7,9834
2	nMS	nL	nMSnL	6,99275
3	nMS	nL	nMSnL	5,271
4	nMS	nL	nMSnL	5,1523
5	nMS	nL	nMSnL	6,5956
6	nMS	nL	nMSnL	1,86525
7	nMS	nL	nMSnL	5,72105
8	nMS	nL	nMSnL	1,772
9	nMS	nL	nMSnL	4,05705
10	nMS	nL	nMSnL	2,7783
11	nMS	nL	nMSnL	1,1352
12	MS	L	MSL	4,8287
13	MS	L	MSL	6,8673
14	MS	L	MSL	4,3521
15	MS	L	MSL	4,9778
16	MS	L	MSL	4,40635
17	MS	L	MSL	9,27825
18	MS	L	MSL	0,4964
19	MS	L	MSL	1,64705
20	MS	L	MSL	5,84945
21	MS	L	MSL	2,0637
22	MS	L	MSL	2,4099
23	MS	L	MSL	3,16145
24	nMS	L	nMSL	7,45685

	Potassium-stimulated [^{3}H]DA release in the NAc core			
	Stress	Treatment	Stress x Treatment	K-Peak
25	nMS	L	nMSL	2,30325
26	nMS	L	nMSL	5,2367
27	nMS	L	nMSL	3,2135
28	nMS	L	nMSL	3,69175
29	nMS	L	nMSL	3,9349
30	nMS	L	nMSL	2,55845
31	nMS	L	nMSL	3,42255
32	nMS	L	nMSL	4,91475
33	nMS	L	nMSL	3,20875
34	MS	nL	MSnL	4,192
35	MS	nL	MSnL	4,5931
36	MS	nL	MSnL	3,92295
37	MS	nL	MSnL	3,5067
38	MS	nL	MSnL	5,21995
39	MS	nL	MSnL	4,5654
40	MS	nL	MSnL	1,2619
41	MS	nL	MSnL	1,16725
42	MS	nL	MSnL	2,846
43	MS	nL	MSnL	4,2714
44	MS	nL	MSnL	2,8139
45	MS	nL	MSnL	1,4342



Breakdown Table of Descriptive Statistics (Statistica Superfusion 31 October 2013)
N=45 (No missing data in dep. var. list)

Stress	Treatment	K-Peak Means	K-Peak N	K-Peak Std.Dev.	K-Peak Std.Err.	K-Peak Q25	K-Peak Median	K-Peak Q75
nMS	nL	4,483991	11	2,326236	0,701386	1,865250	5,152300	6,595600
nMS	L	3,994145	10	1,525708	0,482471	3,208750	3,557150	4,914750
MS	nL	3,316229	12	1,406238	0,405946	2,124050	3,714825	4,418400
MS	L	4,194871	12	2,445227	0,705876	2,236800	4,379225	5,413625
All Groups		3,986634	45	1,972666	0,294068	2,558450	3,934900	5,152300

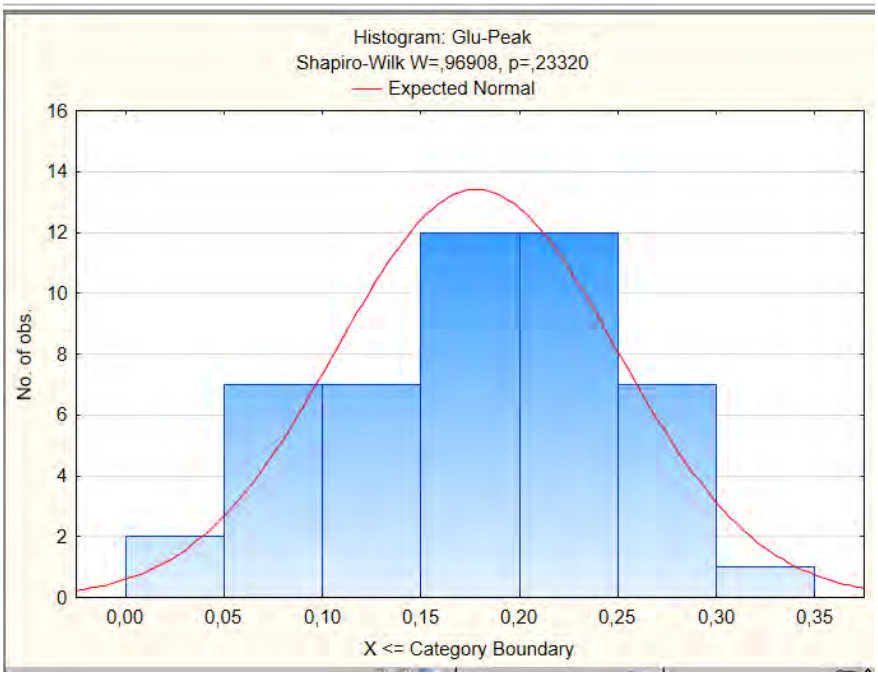
Univariate Tests of Significance for K-Peak (Statistica Superfusion 31 October 2013)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	714,9692	1	714,9692	180,2959	0,000000
Stress	2,6153	1	2,6153	0,6595	0,421426
Treatment	0,4227	1	0,4227	0,1066	0,745705
Stress*Treatment	5,2374	1	5,2374	1,3207	0,257122
Error	162,5868	41	3,9655		

Glutamate-stimulated [^{3}H]DA release in the NAc shell

	Glutamate-stimulated [^{3}H]DA release in the NAc shell			
	Stress	Treatment	Stress x Treatment	Glu-Peak
1	nMS	nL	nMSnL	0,24505
2	nMS	nL	nMSnL	0,272
3	nMS	nL	nMSnL	0,1322
4	nMS	nL	nMSnL	0,29215
5	nMS	nL	nMSnL	0,2011
6	nMS	nL	nMSnL	0,2475
7	nMS	nL	nMSnL	0,168
8	nMS	nL	nMSnL	0,08705
9	nMS	nL	nMSnL	0,24955
10	nMS	nL	nMSnL	0,19785
11	nMS	nL	nMSnL	0,2041
12	MS	L	MSL	0,1061
13	MS	L	MSL	0,06975
14	MS	L	MSL	0,21775
15	MS	L	MSL	0,28195
16	MS	L	MSL	0,2241
17	MS	L	MSL	0,1789
18	MS	L	MSL	0,09325
19	MS	L	MSL	0,141
20	MS	L	MSL	0,1021
21	MS	L	MSL	0,163
22	MS	L	MSL	0,22965
23	MS	L	MSL	0,06095
24	MS	L	MSL	0,03835

	Glutamate-stimulated [^{3}H]DA release in the NAc shell			
	Stress	Treatment	Stress x Treatment	Glu-Peak
25	MS	L	MSL	0,09925
26	nMS	L	nMSL	0,23375
27	nMS	L	nMSL	0,1951
28	nMS	L	nMSL	0,192
29	nMS	L	nMSL	0,1558
30	nMS	L	nMSL	0,0487
31	nMS	L	nMSL	0,26705
32	nMS	L	nMSL	0,31105
33	nMS	L	nMSL	0,25135
34	nMS	L	nMSL	0,0791
35	nMS	L	nMSL	0,15865
36	nMS	L	nMSL	0,11715
37	MS	nL	MSnL	0,0729
38	MS	nL	MSnL	0,2211
39	MS	nL	MSnL	0,21495
40	MS	nL	MSnL	0,10695
41	MS	nL	MSnL	0,2186
42	MS	nL	MSnL	0,194
43	MS	nL	MSnL	0,25845
44	MS	nL	MSnL	0,1894
45	MS	nL	MSnL	0,1459
46	MS	nL	MSnL	0,1609
47	MS	nL	MSnL	0,26725
48	MS	nL	MSnL	0,1896



Breakdown Table of Descriptive Statistics (Statistica Superfusion 31 October 2013)
N=48 (No missing data in dep. var. list)

Stress	Treatment	Glu-Peak Means	Glu-Peak N	Glu-Peak Std.Dev.	Glu-Peak Std.Err.	Glu-Peak Q25	Glu-Peak Median	Glu-Peak Q75
nMS	nL	0,208777	11	0,061620	0,018579	0,168000	0,204100	0,249550
nMS	L	0,182700	11	0,080892	0,024390	0,117150	0,192000	0,251350
MS	nL	0,186667	12	0,057481	0,016593	0,153400	0,191800	0,219850
MS	L	0,143293	14	0,073977	0,019771	0,093250	0,123550	0,217750
All Groups		0,178174	48	0,071358	0,010300	0,112050	0,190800	0,231700

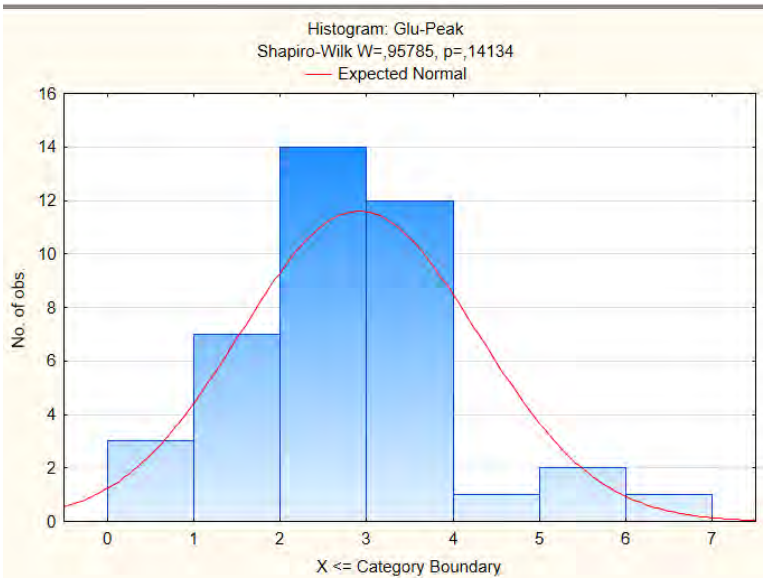
Univariate Tests of Significance for Glu-Peak (Statistica Superfusion 31 October 2013)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	1,546351	1	1,546351	322,6213	0,000000
Stress	0,011244	1	0,011244	2,3458	0,132777
Treatment	0,014331	1	0,014331	2,9899	0,090797
Stress*Treatment	0,000889	1	0,000889	0,1854	0,668837
Error	0,210896	44	0,004793		

Potassium-stimulated [^{3}H]DA release in the NAc shell

	Potassium-stimulated [^{3}H]DA release in the NAc shell			
	Stress	Treatment	Stress x Treatment	K SHELL
1	nMS	nL	nMSnL	6,9009
2	nMS	nL	nMSnL	5,5001
3	nMS	nL	nMSnL	4,8092
4	nMS	nL	nMSnL	3,5549
5	nMS	nL	nMSnL	2,94675
6	nMS	nL	nMSnL	2,6446
7	nMS	nL	nMSnL	2,45055
8	nMS	nL	nMSnL	3,6545
9	nMS	nL	nMSnL	2,5945
10	nMS	nL	nMSnL	2,5516
11	MS	L	MSL	3,7616
12	MS	L	MSL	0,62965
13	MS	L	MSL	3,9375
14	MS	L	MSL	3,9923
15	MS	L	MSL	3,8479
16	MS	L	MSL	1,01295
17	MS	L	MSL	1,817
18	MS	L	MSL	1,78395
19	MS	L	MSL	1,89945
20	MS	L	MSL	1,0693
21	MS	L	MSL	2,77445
22	nMS	L	nMSL	5,8185
23	nMS	L	nMSL	2,1496
24	nMS	L	nMSL	2,9691
25	nMS	L	nMSL	2,698
26	nMS	L	nMSL	2,5498

27	nMS	L	nMSL	1,2351
28	nMS	L	nMSL	0,7807
29	nMS	L	nMSL	3,2371
30	nMS	L	nMSL	2,2131
31	MS	nL	MSnL	3,2881
32	MS	nL	MSnL	3,9886
33	MS	nL	MSnL	3,115
34	MS	nL	MSnL	3,7973
35	MS	nL	MSnL	3,9974
36	MS	nL	MSnL	0,7394
37	MS	nL	MSnL	2,7969
38	MS	nL	MSnL	2,30445
39	MS	nL	MSnL	1,9373
40	MS	nL	MSnL	2,75245



Breakdown Table of Descriptive Statistics (Statistica Superfusion 31 October 2013)
N=40 (No missing data in dep. var. list)

Stress	Treatment	Glu-Peak Means	Glu-Peak N	Glu-Peak Std.Dev.	Glu-Peak Std.Err.	Glu-Peak Q25	Glu-Peak Median	Glu-Peak Q75
nMS	nL	3,760760	10	1,508715	0,477098	2,594500	3,250825	4,809200
nMS	L	2,627889	9	1,433150	0,477717	2,149600	2,549800	2,969100
MS	nL	2,871690	10	1,020097	0,322583	2,304450	2,955950	3,797300
MS	L	2,411459	11	1,295655	0,390655	1,069300	1,899450	3,847900
All Groups		2,912539	40	1,375356	0,217463	2,043450	2,763450	3,779450

Univariate Tests of Significance for Glu-Peak (Statistica Superfusion 31 October 2013)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	338,8657	1	338,8657	193,4228	0,000000
Stress	3,0400	1	3,0400	1,7352	0,196071
Treatment	6,3131	1	6,3131	3,6035	0,065699
Stress*Treatment	1,1254	1	1,1254	0,6424	0,428106
Error	63,0699	36	1,7519		

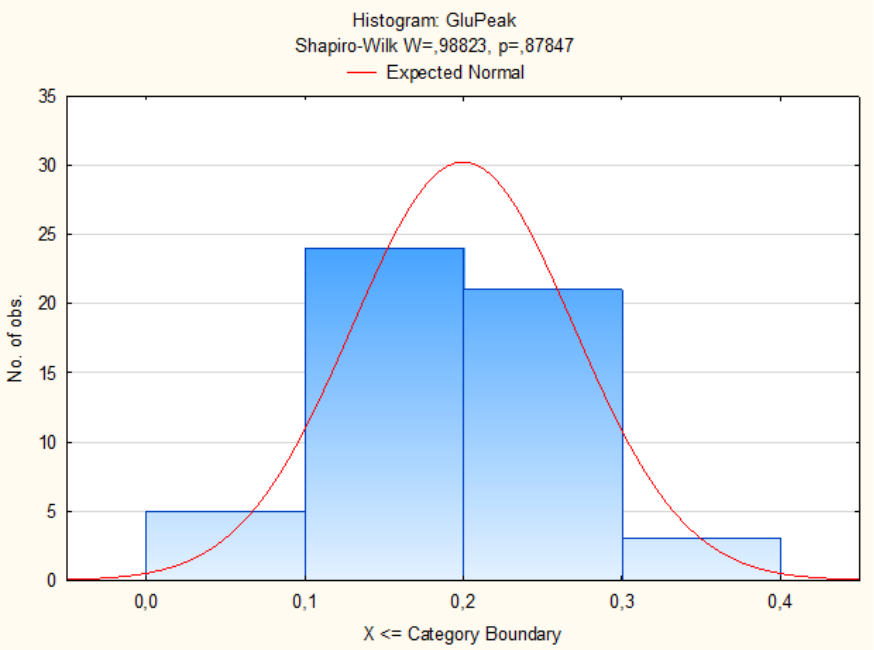
Duncan test; variable K Peak (DATA KSHELL)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = 1,7519, df = 36,000

Cell No.	Stress	Treatment	{1} 3,7608	{2} 2,6279	{3} 2,8717	{4} 2,4115
1	nMS	nL		0,078503	0,142918	0,042943
2	nMS	L	0,078503		0,683756	0,717586
3	MS	nL	0,142918	0,683756		0,471148
4	MS	L	0,042943	0,717586	0,471148	

Glutamate-stimulated [^{3}H]DA release in the NAc

	Glutamate-stimulated [^{3}H]DA release in the NAc			
	Stress	Treatment	Stress x Treatment	GluPeak
1	nMS	nL	nMSnL	0,274
2	nMS	nL	nMSnL	0,24705
3	nMS	nL	nMSnL	0,17025
4	nMS	nL	nMSnL	0,2895
5	nMS	nL	nMSnL	0,3145
6	nMS	nL	nMSnL	0,28195
7	nMS	nL	nMSnL	0,105775
8	nMS	nL	nMSnL	0,176875
9	nMS	nL	nMSnL	0,2136
10	nMS	nL	nMSnL	0,225075
11	nMS	nL	nMSnL	0,2127
12	nMS	nL	nMSnL	0,2536
13	nMS	nL	nMSnL	0,393
14	MS	L	MSL	0,08625
15	MS	L	MSL	0,139625
16	MS	L	MSL	0,18535
17	MS	L	MSL	0,1903
18	MS	L	MSL	0,29155
19	MS	L	MSL	0,172525
20	MS	L	MSL	0,23095
21	MS	L	MSL	0,141
22	MS	L	MSL	0,17825
23	MS	L	MSL	0,1782
24	MS	L	MSL	0,1998
25	MS	L	MSL	0,069525
26	MS	L	MSL	0,08675

	Glutamate-stimulated [^{3}H]DA release in the NAc			
	Stress	Treatment	Stress x Treatment	GluPeak
27	MS	L	MSL	0,247025
28	nMS	L	nMSL	0,2854
29	nMS	L	nMSL	0,23375
30	nMS	L	nMSL	0,19735
31	nMS	L	nMSL	0,175028
32	nMS	L	nMSL	0,0958
33	nMS	L	nMSL	0,0588
34	nMS	L	nMSL	0,325625
35	nMS	L	nMSL	0,25845
36	nMS	L	nMSL	0,234825
37	nMS	L	nMSL	0,175225
38	nMS	L	nMSL	0,18745
39	nMS	L	nMSL	0,15705
40	MS	nL	MSnL	0,1488
41	MS	nL	MSnL	0,136975
42	MS	nL	MSnL	0,212675
43	MS	nL	MSnL	0,101925
44	MS	nL	MSnL	0,153
45	MS	nL	MSnL	0,19435
46	MS	nL	MSnL	0,294325
47	MS	nL	MSnL	0,171075
48	MS	nL	MSnL	0,213675
49	MS	nL	MSnL	0,2107
50	MS	nL	MSnL	0,137525
51	MS	nL	MSnL	0,251
52	MS	nL	MSnL	0,2632
53	MS	nL	MSnL	0,1573



Breakdown Table of Descriptive Statistics (NuACC for STATISTICA 7 November 2013)
N=53 (No missing data in dep. var. list)

Stress	Treatment	GluPeak Means	GluPeak N	GluPeak Std.Dev.	GluPeak Std.Err.	GluPeak Q25	GluPeak Median	GluPeak Q75
nMS	nL	0,242913	13	0,072446	0,020093	0,212700	0,247050	0,281950
nMS	L	0,198729	12	0,075598	0,021823	0,166039	0,192400	0,246638
MS	nL	0,189038	14	0,054974	0,014693	0,148800	0,182713	0,213675
MS	L	0,171221	14	0,063174	0,016884	0,139625	0,178225	0,199800
All Groups		0,199741	53	0,069924	0,009605	0,157050	0,194350	0,247050

Duncan test; variable GluPeak (NuACC for STATISTICA 7 November 2013)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = ,00443, df = 49,000

Cell No.	Stress	Treatment	{1} ,24291	{2} ,19873	{3} ,18904	{4} ,17122
1	nMS	nL		0,094580	0,053807	0,013047
2	nMS	L	0,094580		0,710080	0,323364
3	MS	nL	0,053807	0,710080		0,495046
4	MS	L	0,013047	0,323364	0,495046	

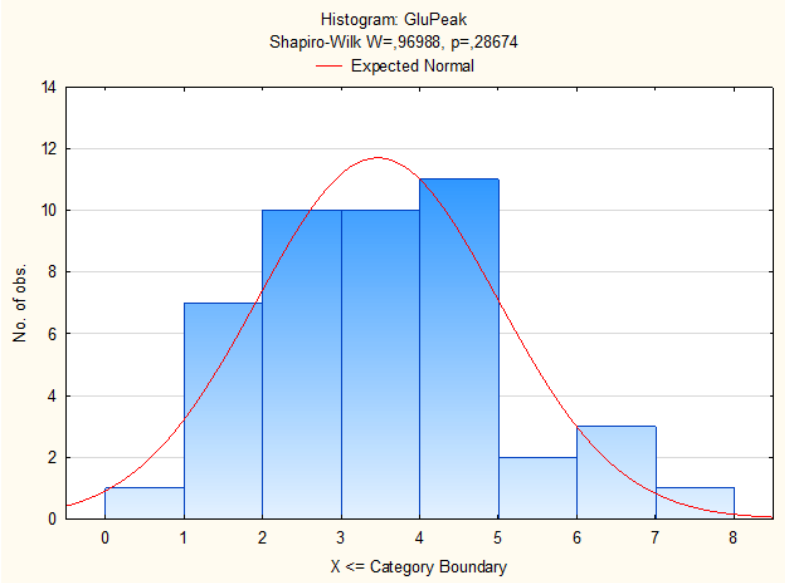
Effect	Univariate Tests of Significance for GluPeak (NuACC for STATISTICA 7 November 2013) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	2,121471	1	2,121471	479,0047	0,000000
Stress	0,021851	1	0,021851	4,9337	0,030990
Treatment	0,012682	1	0,012682	2,8634	0,096966
Stress*Treatment	0,002294	1	0,002294	0,5179	0,475155
Error	0,217017	49	0,004429		

Potassium-stimulated [^{3}H]DA release in the NAc

	Potassium-stimulated [^{3}H]DA release in the NAc			
	Stress	Treatment	Stress x Treatment	GluPeak
1	nMS	nL	nMSnL	7,44215
2	nMS	nL	nMSnL	6,246425
3	nMS	nL	nMSnL	5,0401
4	nMS	nL	nMSnL	4,3536
5	nMS	nL	nMSnL	4,771175
6	nMS	nL	nMSnL	2,254925
7	nMS	nL	nMSnL	4,0858
8	nMS	nL	nMSnL	2,71325
9	nMS	nL	nMSnL	3,325775
10	nMS	nL	nMSnL	2,66495
11	nMS	nL	nMSnL	1,1352
12	MS	L	MSL	4,29515
13	MS	L	MSL	3,748475
14	MS	L	MSL	4,1448
15	MS	L	MSL	4,48505

	Potassium-stimulated [^{3}H]DA release in the NAc			
	Stress	Treatment	Stress x Treatment	GluPeak
16	MS	L	MSL	4,40635
17	MS	L	MSL	6,563075
18	MS	L	MSL	0,754675
19	MS	L	MSL	1,732025
20	MS	L	MSL	3,8167
21	MS	L	MSL	1,981575
22	MS	L	MSL	1,7396
23	MS	L	MSL	2,96795
24	nMS	L	nMSL	6,637675
25	nMS	L	nMSL	2,226425
26	nMS	L	nMSL	5,2367
27	nMS	L	nMSL	3,0913
28	nMS	L	nMSL	3,194875
29	nMS	L	nMSL	3,24235
30	nMS	L	nMSL	1,896775
31	nMS	L	nMSL	2,101625
32	nMS	L	nMSL	4,075925
33	nMS	L	nMSL	2,710925
34	MS	nL	MSnL	3,74005
35	MS	nL	MSnL	4,29085
36	MS	nL	MSnL	3,92295
37	MS	nL	MSnL	3,31085
38	MS	nL	MSnL	4,508625
39	MS	nL	MSnL	4,2814
40	MS	nL	MSnL	1,00065
41	MS	nL	MSnL	1,16725
42	MS	nL	MSnL	2,82145

	Potassium-stimulated [^{3}H]DA release in the NAc			
	Stress	Treatment	Stress x Treatment	GluPeak
43	MS	nL	MSnL	3,287925
44	MS	nL	MSnL	2,3756
45	MS	nL	MSnL	2,093325



Breakdown Table of Descriptive Statistics (NuACC for STATISTICA 7 November 2013)
N=45 (No missing data in dep. var. list)

Stress	Treatment	GluPeak Means	GluPeak N	GluPeak Std.Dev.	GluPeak Std.Err.	GluPeak Q25	GluPeak Median	GluPeak Q75
nMS	nL	4,003032	11	1,839801	0,554721	2,664950	4,085800	5,040100
nMS	L	3,441458	10	1,500203	0,474406	2,226425	3,143088	4,075925
MS	nL	3,066744	12	1,195066	0,344986	2,234463	3,299388	4,102175
MS	L	3,386285	12	1,611870	0,465307	1,860588	3,782588	4,350750
All Groups		3,464095	45	1,534163	0,228700	2,254925	3,310850	4,295150

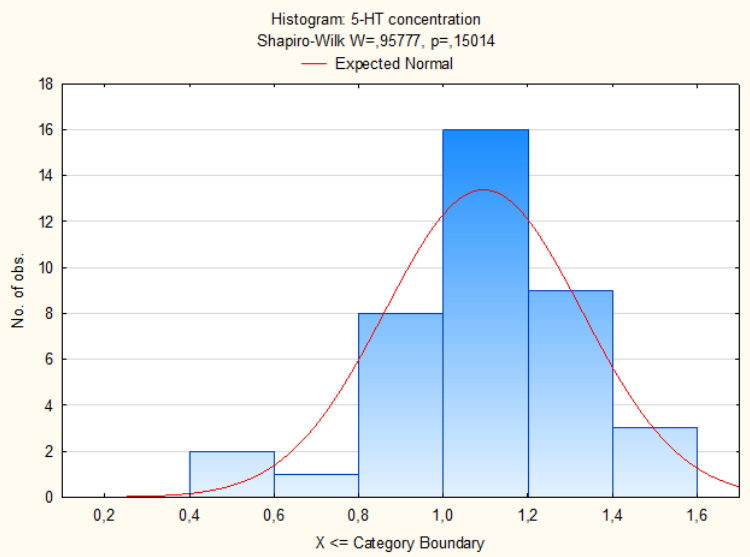
Univariate Tests of Significance for GluPeak (NuACC for STATISTICA 7 November 2013)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	540,1401	1	540,1401	225,0732	0,000000
Stress	2,7490	1	2,7490	1,1455	0,290751
Treatment	0,1638	1	0,1638	0,0683	0,795188
Stress*Treatment	2,1712	1	2,1712	0,9047	0,347092
Error	98,3935	41	2,3998		

Appendix H2: 5-HT concentration in the hypothalamus

	5HT CONCENTRATION (Hypothalamus)			
	Stress	Treatment	Stress Xtreatment	5-HT concentration
1	nMS	nL	nMSnL	1,16951
2	nMS	nL	nMSnL	0,564532
3	nMS	nL	nMSnL	0,702776
4	nMS	nL	nMSnL	1,001948
5	nMS	nL	nMSnL	0,947592
6	nMS	nL	nMSnL	0,939888
7	nMS	nL	nMSnL	1,12457
8	nMS	nL	nMSnL	1,09675
9	nMS	nL	nMSnL	1,115368
10	nMS	L	nMSl	1,01757
11	nMS	L	nMSl	0,429284
12	nMS	L	nMSl	1,11173
13	nMS	L	nMSl	1,06358
14	nMS	L	nMSl	1,201824
15	nMS	L	nMSl	1,552142
16	nMS	L	nMSl	1,318454
17	nMS	L	nMSl	1,204392
18	nMS	L	nMSl	1,156456
19	nMS	L	nMSl	1,145756
20	MS	nL	MSnL	0,869482
21	MS	nL	MSnL	0,828394
22	MS	nL	MSnL	1,102528
23	MS	nL	MSnL	0,925122
24	MS	nL	MSnL	0,963642
25	MS	nL	MSnL	0,925122
26	MS	nL	MSnL	1,105952
27	MS	nL	MSnL	1,177856

	5HT CONCENTRATION (Hypothalamus)			
	Stress	Treatment	Stress Xtreatment	5-HT concentration
28	MS	nL	MSnL	1,18021
29	MS	nL	MSnL	0,906076
30	MS	L	MSL	1,023134
31	MS	L	MSL	1,20803
32	MS	L	MSL	1,529886
33	MS	L	MSL	1,198186
34	MS	L	MSL	1,280576
35	MS	L	MSL	1,212738
36	MS	L	MSL	1,284428
37	MS	L	MSL	1,383082
38	MS	L	MSL	1,24227
39	MS	L	MSL	1,500782



Breakdown Table of Descriptive Statistics (16 SEP 5HT CONCENTRATION STATS)
N=39 (No missing data in dep. var. list)

Stress	Treatment	5-HT concentration Means	5-HT concentration N	5-HT concentration Std.Dev.	5-HT concentration Std.Err.
nMS	nL	0,962548	9	0,205741	0,068580
nMS	L	1,120119	10	0,285340	0,090233
MS	nL	0,998438	10	0,130666	0,041320
MS	L	1,286311	10	0,151010	0,047754
All Groups		1,095170	39	0,232478	0,037226

Univariate Tests of Significance for 5-HT concentration (16 SEP 5HT CONCENTRATION STATS)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	46,39701	1	46,39701	1135,347	0,000000
Stress	0,09933	1	0,09933	2,431	0,127975
Treatment	0,48264	1	0,48264	11,810	0,001535
Stress*Treatment	0,04130	1	0,04130	1,011	0,321657
Error	1,43031	35	0,04087		

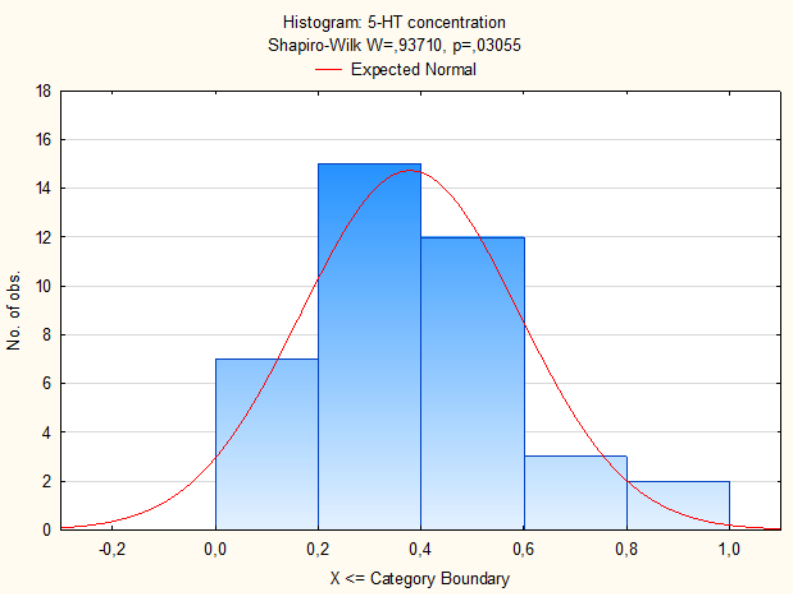
Duncan test; variable 5-HT concentration (16 SEP 5HT CONCENTRATION STATS)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = ,04087, df = 35,000

Cell No.	Stress	Treatment	{1} ,96255	{2} 1,1201	{3} ,99844	{4} 1,2863
1	nMS	nL		0,112567	0,697869	0,002101
2	nMS	L	0,112567		0,193001	0,078495
3	MS	nL	0,697869	0,193001		0,004732
4	MS	L	0,002101	0,078495	0,004732	

Appendix H3: 5-HT concentration in the PFC

	5HT CONCENTRATION (PFC)			
	Stress	Treatment	Stress Xtreatment	5-HT concentration
1	nMS	nL	nMSnL	0,471656
2	nMS	nL	nMSnL	0,907788
3	nMS	nL	nMSnL	0,792442
4	nMS	nL	nMSnL	0,456034
5	nMS	nL	nMSnL	0,610328
6	nMS	nL	nMSnL	0,958506
7	nMS	nL	nMSnL	0,462454
8	nMS	nL	nMSnL	0,373216
9	nMS	nL	nMSnL	0,4708
10	nMS	nL	nMSnL	0,417728
11	nMS	L	nMSL	0,402106
12	nMS	L	nMSL	0,359734
13	nMS	L	nMSL	0,308802
14	nMS	L	nMSL	0,442552
15	nMS	L	nMSL	0,267928
16	nMS	L	nMSL	0,124762
17	nMS	L	nMSL	0,047722
18	nMS	L	nMSL	0,200732
19	nMS	L	nMSL	0,206296
20	MS	nL	MSnL	0,3103
21	MS	nL	MSnL	0,49862
22	MS	nL	MSnL	0,40125
23	MS	nL	MSnL	0,406386
24	MS	nL	MSnL	0,695928
25	MS	nL	MSnL	0,376426
26	MS	nL	MSnL	0,472512
27	MS	nL	MSnL	0,349034

	5HT CONCENTRATION (PFC)			
	Stress	Treatment	Stress Xtreatment	5-HT concentration
28	MS	nL	MSnL	0,29639
29	MS	nL	MSnL	0,386698
30	MS	L	MSL	0,268784
31	MS	L	MSL	0,217424
32	MS	L	MSL	0,27927
33	MS	L	MSL	0,33919
34	MS	L	MSL	0,578014
35	MS	L	MSL	0,054998
36	MS	L	MSL	0,122408
37	MS	L	MSL	0,19581
38	MS	L	MSL	0,18083
39	MS	L	MSL	0,069764



Breakdown Table of Descriptive Statistics (16 SEP 5HT CONCENTRATION STATS)
N=39 (No missing data in dep. var. list)

Treatment	Stress	5-HT concentration Means	5-HT concentration N	5-HT concentration Std.Dev.	5-HT concentration Q25	5-HT concentration Median	5-HT concentration Q75
nL	nMS	0,592095	10	0,215314	0,456034	0,471228	0,792442
nL	MS	0,419354	10	0,115933	0,349034	0,393974	0,472512
L	nMS	0,262293	9	0,130198	0,200732	0,267928	0,359734
L	MS	0,230649	10	0,152237	0,122408	0,206617	0,279270
All Groups		0,379016	39	0,211227	0,217424	0,373216	0,470800

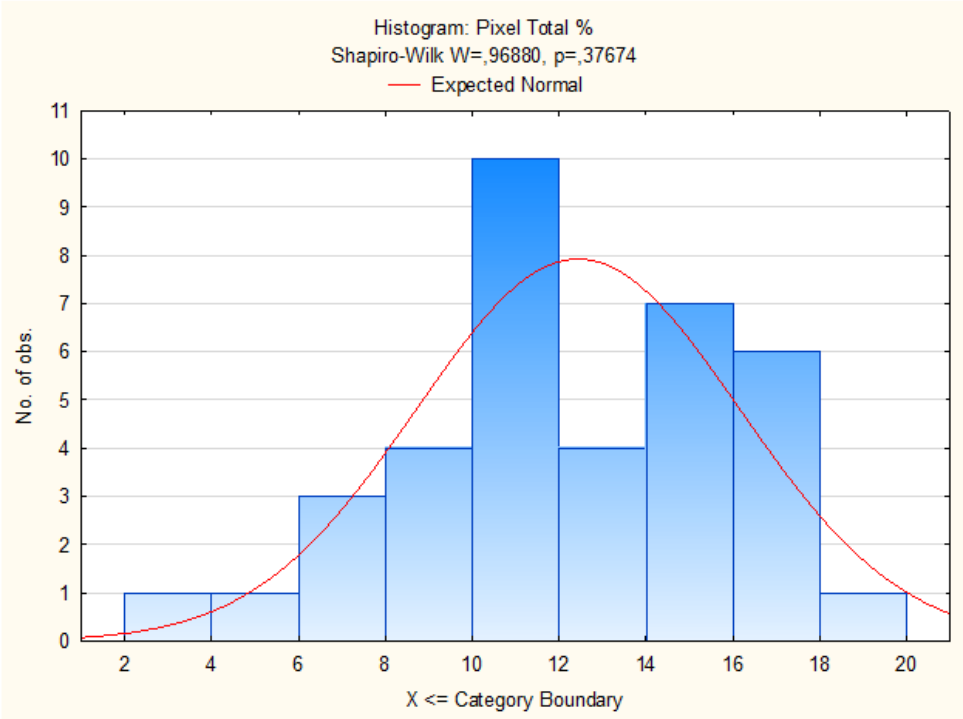
Multiple Comparisons p values (2-tailed); 5-HT concentration (16 SEP 5HT CONCENTRATION STATS)
Independent (grouping) variable: Stress Xtreatment
Kruskal-Wallis test: H (3, N= 39) =20,74419 p =,0001

Depend.: 5-HT concentration	nMSnL R:31,300	nMSL R:13,222	MSnL R:24,000	MSL R:10,800
nMSnL		0,003354	0,913468	0,000349
nMSL	0,003354		0,237932	1,000000
MSnL	0,913468	0,237932		0,057798
MSL	0,000349	1,000000	0,057798	

Appendix H4: MOR-1 protein levels in the NAc core

	MOR-1 protein levels in the NAc core			
	Stress	Treatment	Stress x Treatment	Pixel Total %
1	nMS	nL	nMSnL	13,57
2	nMS	nL	nMSnL	11,46
3	nMS	nL	nMSnL	14,9082696
4	nMS	nL	nMSnL	10,6659039
5	nMS	nL	nMSnL	13,3
6	nMS	nL	nMSnL	17,29
7	nMS	nL	nMSnL	8,03545877
8	nMS	nL	nMSnL	5,90612322
9	nMS	nL	nMSnL	13,207752
10	nMS	nL	nMSnL	16,8629916
11	nMS	L	nMSL	11,96
12	nMS	L	nMSL	10,56
13	nMS	L	nMSL	11,23026
14	nMS	L	nMSL	15,975963
15	nMS	L	nMSL	8,08
16	nMS	L	nMSL	15,5
17	nMS	L	nMSL	17,9353626
18	nMS	L	nMSL	10,4946121
19	nMS	L	nMSL	16,7916431
20	MS	nL	MSnL	9,1
21	MS	nL	MSnL	16,32
22	MS	nL	MSnL	14,8852016
23	MS	nL	MSnL	11,42
24	MS	nL	MSnL	11,99
25	MS	nL	MSnL	16,8830843
26	MS	nL	MSnL	14,8914216

27	MS	nL	MSnL	7,60807589
28	MS	nL	MSnL	18,6270906
29	MS	L	MSL	11,89
30	MS	L	MSL	15,14
31	MS	L	MSL	11,9350812
32	MS	L	MSL	7,89932075
33	MS	L	MSL	6,53
34	MS	L	MSL	15,89
35	MS	L	MSL	8,63271355
36	MS	L	MSL	12,8593152
37	MS	L	MSL	3,95745024



Breakdown Table of Descriptive Statistics (MOR1 EXCEL 03 MARCH 2014)
N=37 (No missing data in dep. var. list)

Stress	Treatment	Pixel Total % Means	Pixel Total % N	Pixel Total % Std.Dev.	Pixel Total % Std.Err.
nMS	nL	12,52065	10	3,619668	1,144639
nMS	L	13,16976	9	3,432053	1,144018
MS	nL	13,52499	9	3,711725	1,237242
MS	L	10,52599	9	4,014812	1,338271
All Groups		12,43765	37	3,725805	0,612519

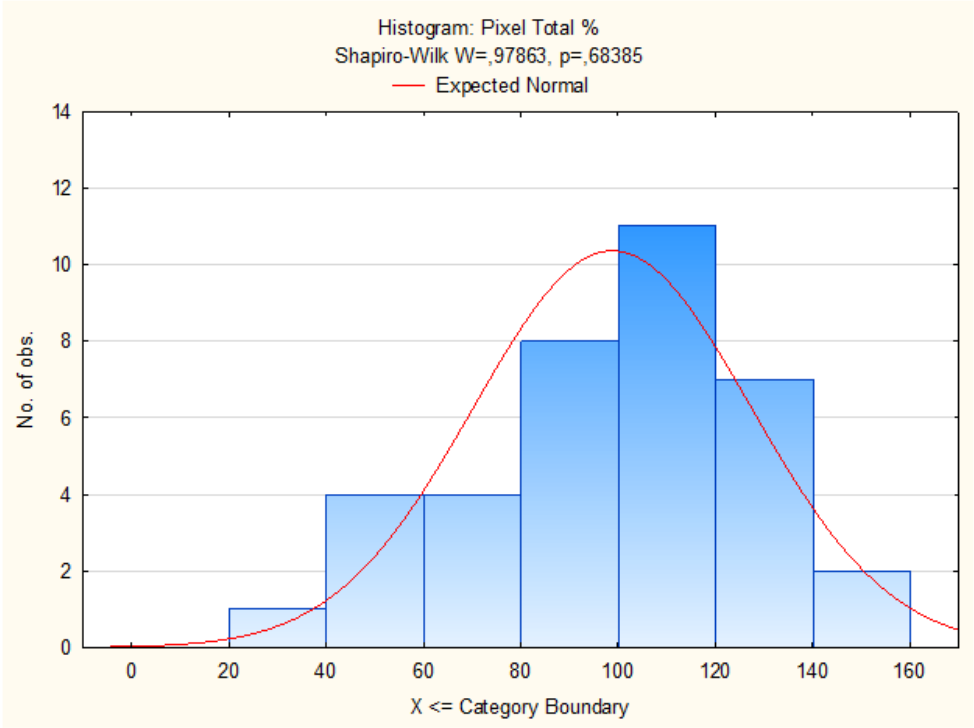
Univariate Tests of Significance for Pixel Total % (MOR1 EXCEL 03 MARCH 2014)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	5709,704	1	5709,704	417,4919	0,000000
Stress	6,203	1	6,203	0,4535	0,505355
Treatment	12,743	1	12,743	0,9318	0,341425
Stress*Treatment	30,712	1	30,712	2,2457	0,143494
Error	451,315	33	13,676		

MOR-1 protein levels in the NAc core (normalised against α-tubulin)

	MOR-1 protein levels in the NAc core normalised			
	Stress	Treatment	Stress x Treatment	Pixel Total %
1	nMS	nL	nMSnL	122,112805
2	nMS	nL	nMSnL	106,474375
3	nMS	nL	nMSnL	113,284723
4	nMS	nL	nMSnL	95,6583313
5	nMS	nL	nMSnL	80,1204819
6	nMS	nL	nMSnL	137,331215
7	nMS	nL	nMSnL	76,6011322
8	nMS	nL	nMSnL	51,3129733
9	nMS	nL	nMSnL	97,9803559
10	nMS	nL	nMSnL	108,391152
11	nMS	L	nMSL	85,9613236
12	nMS	L	nMSL	91,8453559
13	nMS	L	nMSL	102,186169
14	nMS	L	nMSL	123,940753
15	nMS	L	nMSL	63,6721828
16	nMS	L	nMSL	142,332415
17	nMS	L	nMSL	125,422116
18	nMS	L	nMSL	93,0373411
19	nMS	L	nMSL	119,683843
20	MS	nL	MSnL	68,2108123
21	MS	nL	MSnL	123,892095
22	MS	nL	MSnL	118,986423
23	MS	nL	MSnL	82,8136331
24	MS	nL	MSnL	108,31075
25	MS	nL	MSnL	121,987603
26	MS	nL	MSnL	107,519289
27	MS	nL	MSnL	59,7649324

28	MS	nL	MSnL	131,919905
29	MS	L	MSL	93,5510508
30	MS	L	MSL	112,231573
31	MS	L	MSL	111,024011
32	MS	L	MSL	52,3133825
33	MS	L	MSL	53,3061224
34	MS	L	MSL	157,01581
35	MS	L	MSL	79,3447937
36	MS	L	MSL	105,5773
37	MS	L	MSL	30,9546474



Breakdown Table of Descriptive Statistics (MOR1 and ALPHA 4 MARCH 2014)
N=37 (No missing data in dep. var. list)

Stress	Treatment	Pixel Total % Means	Pixel Total % N	Pixel Total % Std.Dev.	Pixel Total % Std.Err.	Pixel Total % Q25	Pixel Total % Median	Pixel Total % Q75
nMS	nL	98,9268	10	24,71155	7,81448	80,12048	102,2274	113,2847
nMS	L	105,3424	9	24,44026	8,14675	91,84536	102,1862	123,9408
MS	nL	102,6006	9	26,03645	8,67882	82,81363	108,3107	121,9876
MS	L	88,3687	9	38,77063	12,92354	53,30612	93,5511	111,0240
All Groups		98,8128	37	28,48865	4,68350	80,12048	105,5773	119,6838

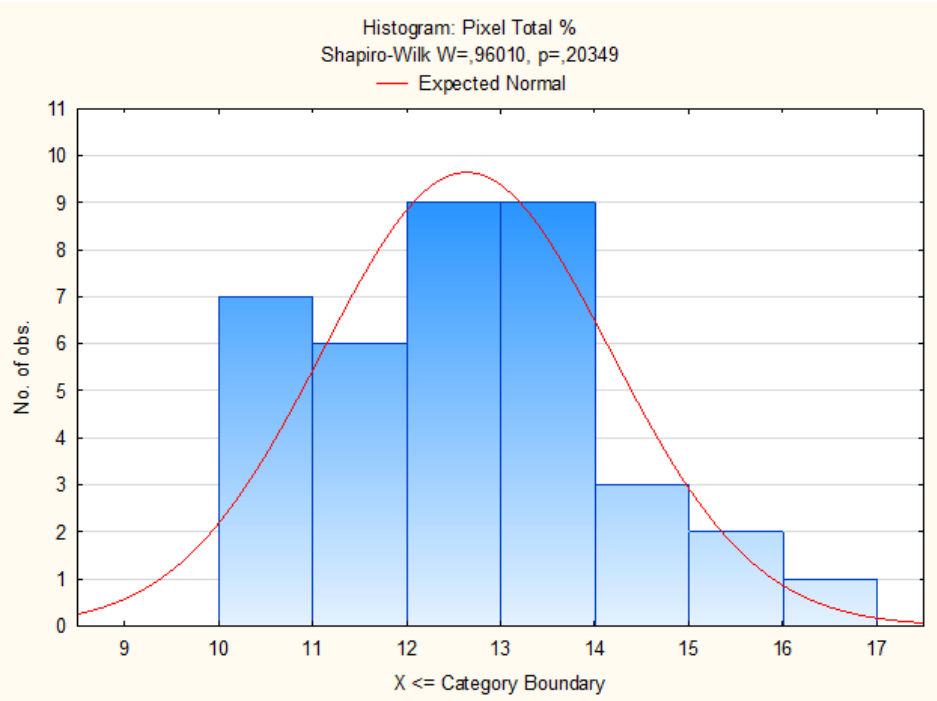
Univariate Tests of Significance for Pixel Total % (MOR1 and ALPHA 4 MARCH 2014)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	360492,6	1	360492,6	429,1110	0,000000
Stress	408,2	1	408,2	0,4859	0,490647
Treatment	141,0	1	141,0	0,1678	0,684703
Stress*Treatment	983,8	1	983,8	1,1711	0,287024
Error	27723,0	33	840,1		

Alpha tubulin protein levels in the NAc core

	Alpha tubulin protein levels in the NAc core			
	Stress	Treatment	Stress x treatment	Pixel Total %
1	nMS	nL	nMSnL	11,1126757
2	nMS	nL	nMSnL	10,7631531
3	nMS	nL	nMSnL	13,16
4	nMS	nL	nMSnL	11,15
5	nMS	nL	nMSnL	16,6
6	nMS	nL	nMSnL	12,59
7	nMS	nL	nMSnL	10,49
8	nMS	nL	nMSnL	11,51
9	nMS	nL	nMSnL	13,48
10	nMS	nL	nMSnL	15,5575352
11	nMS	L	nMSL	13,9132339
12	nMS	L	nMSL	11,4975873
13	nMS	L	nMSL	10,99
14	nMS	L	nMSL	12,89
15	nMS	L	nMSL	12,69
16	nMS	L	nMSL	10,89
17	nMS	L	nMSL	14,3
18	nMS	L	nMSL	11,28
19	nMS	L	nMSL	14,03
20	MS	nL	MSnL	13,3409935
21	MS	nL	MSnL	13,1727533
22	MS	nL	MSnL	12,51
23	MS	nL	MSnL	13,79
24	MS	nL	MSnL	11,07
25	MS	nL	MSnL	13,84
26	MS	nL	MSnL	13,85

27	MS	nL	MSnL	12,73
28	MS	nL	MSnL	14,12
29	MS	L	MSL	12,7096381
30	MS	L	MSL	13,4899651
31	MS	L	MSL	10,75
32	MS	L	MSL	15,1
33	MS	L	MSL	12,25
34	MS	L	MSL	10,12
35	MS	L	MSL	10,88
36	MS	L	MSL	12,18
37	MS	L	MSL	12,7846723



Breakdown Table of Descriptive Statistics (Alpha Tubulin ONLY 4 MARCH 2014)
N=37 (No missing data in dep. var. list)

Stress	Treatment	Pixel Total % Means	Pixel Total % N	Pixel Total % Std.Dev.	Pixel Total % Std.Err.
nMS	nL	12,64134	10	2,085582	0,659519
nMS	L	12,49787	9	1,375305	0,458435
MS	nL	13,15819	9	0,952982	0,317661
MS	L	12,25159	9	1,534024	0,511341
All Groups		12,63736	37	1,530486	0,251610

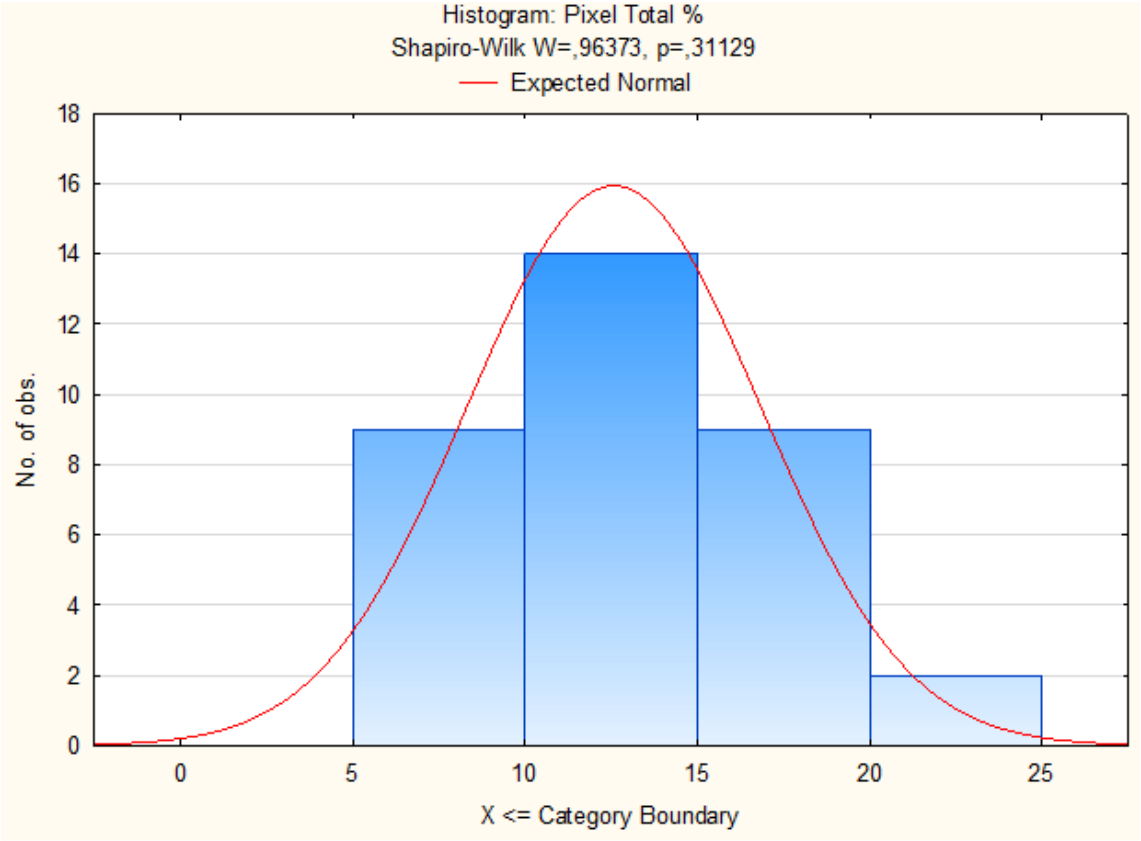
Univariate Tests of Significance for Pixel Total % (Alpha Tubulin ONLY 4 MARCH 2014)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	5896,615	1	5896,615	2421,162	0,000000
Stress	0,169	1	0,169	0,069	0,793893
Treatment	2,545	1	2,545	1,045	0,314139
Stress*Treatment	1,344	1	1,344	0,552	0,462825
Error	80,370	33	2,435		

Appendix H5: MOR-1 protein levels in the NAc shell

	MOR-1 protein levels in the NAc shell			
	Stress	Treatment	Stress x Treatment	Pixel Total %
1	nMS	nL	nMSnL	11,3544746
2	nMS	nL	nMSnL	15,8963433
3	nMS	nL	nMSnL	5,6898358
4	nMS	nL	nMSnL	15,6187939
5	nMS	nL	nMSnL	8,91313277
6	nMS	nL	nMSnL	9,14427415
7	nMS	nL	nMSnL	23,5568667
8	nMS	nL	nMSnL	11,65
9	nMS	nL	nMSnL	7,85
10	nMS	L	nMSL	12,0263667
11	nMS	L	nMSL	6,89726002
12	nMS	L	nMSL	19,1819735
13	nMS	L	nMSL	11,2211645
14	nMS	L	nMSL	15,7618579
15	nMS	L	nMSL	16,75
16	nMS	L	nMSL	12,76
17	MS	nL	MSnL	16,2197326
18	MS	nL	MSnL	12,7633192
19	MS	nL	MSnL	15,1849501
20	MS	nL	MSnL	18,601239
21	MS	nL	MSnL	11,6267616
22	MS	nL	MSnL	13,2098364
23	MS	nL	MSnL	12,1929618
24	MS	nL	MSnL	15,757251
25	MS	nL	MSnL	8,42
26	MS	nL	MSnL	9,29

27	MS	L	MSL	10,136107
28	MS	L	MSL	11,0473023
29	MS	L	MSL	7,36592122
30	MS	L	MSL	10,5396751
31	MS	L	MSL	11,8299423
32	MS	L	MSL	6,02820446
33	MS	L	MSL	21,18
34	MS	L	MSL	12,1



Breakdown Table of Descriptive Statistics (MOR1 EXCEL 03 MARCH 2014)
N=34 (No missing data in dep. var. list)

Stress	Treatment	Pixel Total % Means	Pixel Total % N	Pixel Total % Std.Dev.	Pixel Total % Std.Err.
nMS	nL	12,18597	9	5,440483	1,813494
nMS	L	13,51409	7	4,071658	1,538942
MS	nL	13,32661	10	3,169787	1,002375
MS	L	11,27839	8	4,532374	1,602436
All Groups		12,58134	34	4,253764	0,729514

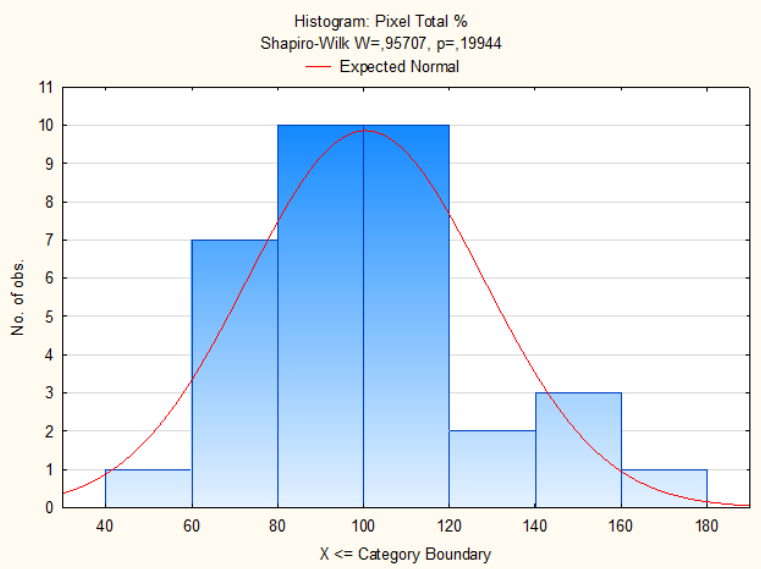
Univariate Tests of Significance for Pixel Total % (MOR1 EXCEL 03 MARCH 2014)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	5283,437	1	5283,437	277,8387	0,000000
Stress	2,504	1	2,504	0,1317	0,719264
Treatment	1,083	1	1,083	0,0569	0,813037
Stress*Treatment	23,800	1	23,800	1,2516	0,272129
Error	570,486	30	19,016		

MOR1 protein levels in the NAc shell normalised against α tubulin

	MOR1 protein levels in the shell (normalised)			
	Stress	Treatment	Stress x Treatment	Pixel Total %
1	nMS	nL	nMSnL	90,9085233
2	nMS	nL	nMSnL	101,698554
3	nMS	nL	nMSnL	56,4810344
4	nMS	nL	nMSnL	118,729773
5	nMS	nL	nMSnL	77,7404482
6	nMS	nL	nMSnL	83,4430215
7	nMS	nL	nMSnL	171,623058
8	nMS	nL	nMSnL	81,0716771
9	nMS	nL	nMSnL	68,6789151
10	nMS	L	nMSL	100,219722
11	nMS	L	nMSL	69,8933789
12	nMS	L	nMSL	120,315749
13	nMS	L	nMSL	96,0711158
14	nMS	L	nMSL	106,259747
15	nMS	L	nMSL	158,31758
16	nMS	L	nMSL	106,688963
17	MS	nL	MSnL	115,525161
18	MS	nL	MSnL	104,360746
19	MS	nL	MSnL	118,438567
20	MS	nL	MSnL	128,792734
21	MS	nL	MSnL	83,0910791
22	MS	nL	MSnL	116,042596
23	MS	nL	MSnL	91,0371232
24	MS	nL	MSnL	142,247266
25	MS	nL	MSnL	70,519263
26	MS	nL	MSnL	71,3517665
27	MS	L	MSL	97,1822338
28	MS	L	MSL	94,5125684
29	MS	L	MSL	71,1581049

30	MS	L	MSL	88,2875051
31	MS	L	MSL	110,228347
32	MS	L	MSL	60,2676243
33	MS	L	MSL	148,735955
34	MS	L	MSL	97,1107544



Breakdown Table of Descriptive Statistics (MOR1 and ALPHA 4 MARCH 2014)
N=34 (No missing data in dep. var. list)

Stress	Treatment	Pixel Total % Means	Pixel Total % N	Pixel Total % Std.Dev.	Pixel Total % Std.Err.	Pixel Total % Q25	Pixel Total % Median	Pixel Total % Q75
nMS	nL	94,4861	9	34,08701	11,36234	77,74045	83,4430	101,6986
nMS	L	108,2523	7	26,90348	10,16856	96,07112	106,2597	120,3157
MS	nL	104,1406	10	24,39462	7,71426	83,09108	109,9430	118,4386
MS	L	95,9354	8	26,56997	9,39390	79,72281	95,8117	103,7053
All Groups		100,5009	34	27,51375	4,71857	81,07168	97,1465	116,0426

Univariate Tests of Significance for Pixel Total % (MOR1 and ALPHA 4 MARCH 2014)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	338768,8	1	338768,8	424,5968	0,000000
Stress	14,8	1	14,8	0,0185	0,892577
Treatment	64,6	1	64,6	0,0809	0,778006
Stress*Treatment	1007,9	1	1007,9	1,2632	0,269953
Error	23935,8	30	797,9		

Alpha tubulin protein levels in the NAc shell

	Alpha tubulin protein levels in the NAc shell			
	Stress	Treatment	Stress x Treatment	Total Pixel (%)
1	nMS	nL	nMSnL	12,49
2	nMS	nL	nMSnL	15,6308449
3	nMS	nL	nMSnL	10,0738874
4	nMS	nL	nMSnL	13,1549092
5	nMS	nL	nMSnL	11,4652449
6	nMS	nL	nMSnL	10,9587045
7	nMS	nL	nMSnL	13,7259334
8	nMS	nL	nMSnL	14,37
9	nMS	nL	nMSnL	11,43
10	nMS	L	nMSL	12
11	nMS	L	nMSL	9,86825952
12	nMS	L	nMSL	15,943028
13	nMS	L	nMSL	11,6800606
14	nMS	L	nMSL	14,833329
15	nMS	L	nMSL	10,58
16	nMS	L	nMSL	11,96
17	MS	nL	MSnL	14,04
18	MS	nL	MSnL	12,23
19	MS	nL	MSnL	12,8209506
20	MS	nL	MSnL	14,4427706
21	MS	nL	MSnL	13,9927917
22	MS	nL	MSnL	11,3836098
23	MS	nL	MSnL	13,3933954
24	MS	nL	MSnL	11,0773665
25	MS	nL	MSnL	11,94

26	MS	nL	MSnL	13,02
27	MS	L	MSL	10,43
28	MS	L	MSL	11,6887124
29	MS	L	MSL	10,3514859
30	MS	L	MSL	11,9379011
31	MS	L	MSL	10,7322142
32	MS	L	MSL	10,0023927
33	MS	L	MSL	14,24
34	MS	L	MSL	12,46

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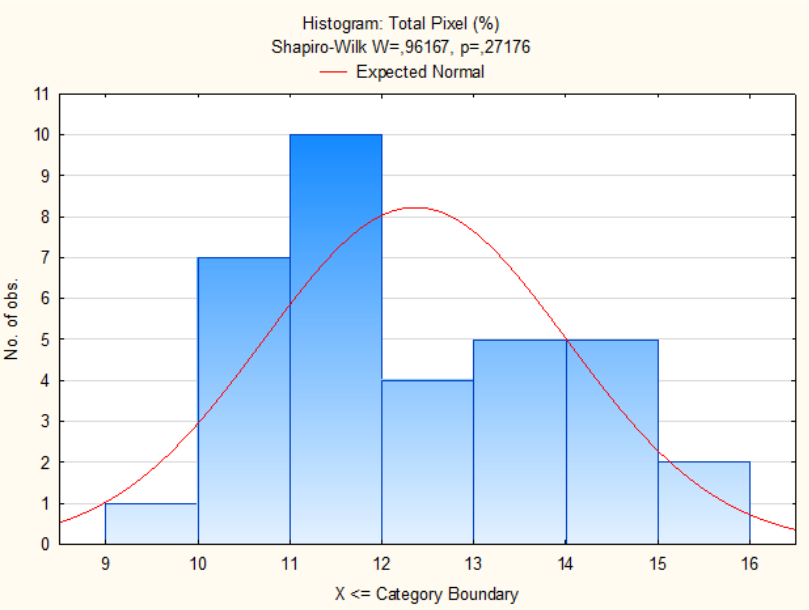


Breakdown Table of Descriptive Statistics (Alpha Tubulin ONLY 4 MARCH 2014)
N=34 (No missing data in dep. var. list)

Stress	Treatment	Total Pixel (%) Means	Total Pixel (%) N	Total Pixel (%) Std.Dev.	Total Pixel (%) Std.Err.
nMS	nL	12,58884	9	1,790154	0,596718
nMS	L	12,40924	7	2,201044	0,831916
MS	nL	12,83409	10	1,158855	0,366462
MS	L	11,48034	8	1,411675	0,499102
All Groups		12,36317	34	1,648388	0,282696

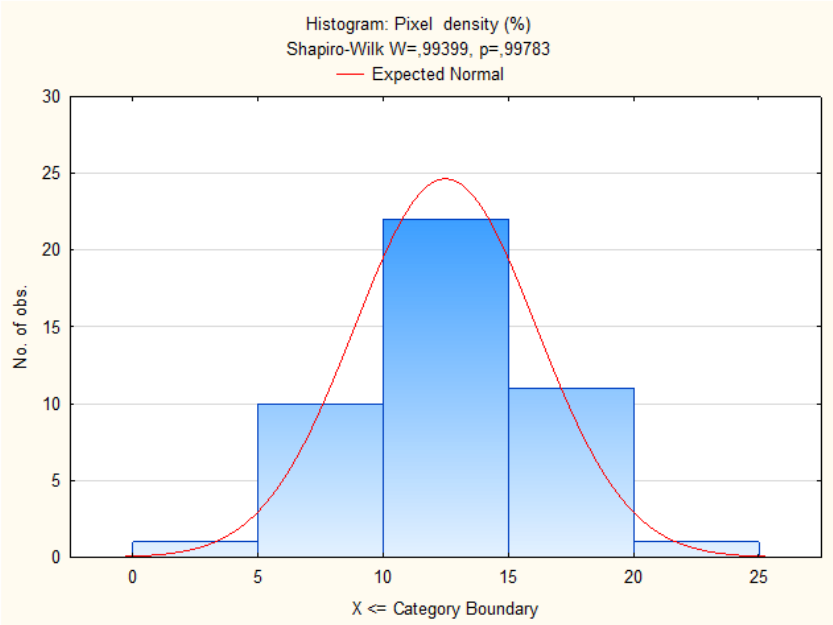
Univariate Tests of Significance for Total Pixel (%) (Alpha Tubulin ONLY 4 MARCH 2014)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	5077,002	1	5077,002	1886,401	0,000000
Stress	0,976	1	0,976	0,363	0,551609
Treatment	4,909	1	4,909	1,824	0,186950
Stress*Treatment	2,878	1	2,878	1,069	0,309329
Error	80,741	30	2,691		

MOR-1 protein levels in the NAc

	MOR-1 protein levels in the nucleus accumbens			
	STRESS	TREATMENT	STRESS X TREATMENT	Pixel density (%)
1	nMS	nL	nMSnL	18,5634333
2	nMS	nL	nMSnL	11,7431833
3	nMS	nL	nMSnL	15,4023064
4	nMS	nL	nMSnL	11,157952
5	nMS	nL	nMSnL	13,0867321
6	nMS	nL	nMSnL	6,86264729
7	nMS	nL	nMSnL	17,29
8	nMS	nL	nMSnL	16,8629916
9	nMS	nL	nMSnL	11,3544746
10	nMS	nL	nMSnL	15,6187939
11	nMS	nL	nMSnL	8,91313277
12	nMS	nL	nMSnL	9,14427415
13	nMS	nL	nMSnL	7,85
14	nMS	L	nMSL	9,06375999
15	nMS	L	nMSL	10,42
16	nMS	L	nMSL	13,3605823
17	nMS	L	nMSL	17,3426813
18	nMS	L	nMSL	11,96
19	nMS	L	nMSL	10,56
20	nMS	L	nMSL	15,975963
21	nMS	L	nMSL	10,4946121
22	nMS	L	nMSL	16,7916431
23	nMS	L	nMSL	19,1819735
24	nMS	L	nMSL	15,7618579
25	MS	nL	MSnL	9,61805349
26	MS	nL	MSnL	12,37
27	MS	nL	MSnL	13,5874751

28	MS	nL	MSnL	14,254923
29	MS	nL	MSnL	13,5421917
30	MS	nL	MSnL	13,1922991
31	MS	nL	MSnL	14,8852016
32	MS	nL	MSnL	11,42
33	MS	nL	MSnL	12,7633192
34	MS	nL	MSnL	9,29
35	MS	L	MSL	6,94796061
36	MS	L	MSL	13,4686512
37	MS	L	MSL	7,33045901
38	MS	L	MSL	11,89
39	MS	L	MSL	15,14
40	MS	L	MSL	11,9350812
41	MS	L	MSL	7,89932075
42	MS	L	MSL	3,95745024
43	MS	L	MSL	10,5396751
44	MS	L	MSL	21,18
45	MS	L	MSL	12,1



Breakdown Table of Descriptive Statistics (27 May 2014 MOR1 Nucleus Acc)
N=45 (No missing data in dep. var. list)

STRESS	TREATMENT	Pixel density (%) Means	Pixel density (%) N	Pixel density (%) Std.Dev.	Pixel density (%) Std.Err.
nMS	nL	12,60384	13	3,854804	1,069130
nMS	L	13,71937	11	3,432734	1,035008
MS	nL	12,49235	10	1,867225	0,590468
MS	L	11,12624	11	4,678414	1,410595
All Groups		12,49056	45	3,642105	0,542933

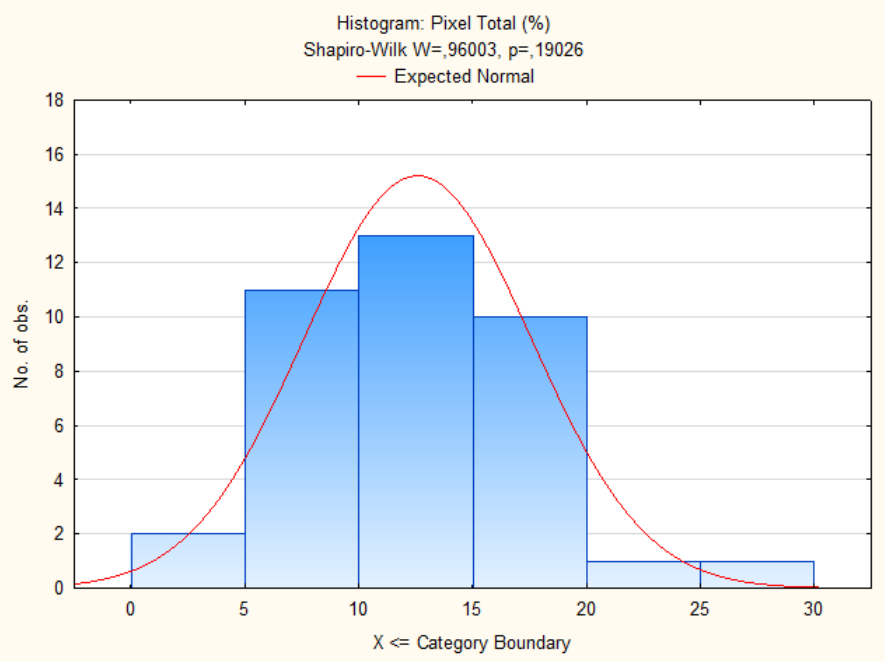
Univariate Tests of Significance for Pixel density (%) (27 May 2014 MOR1 Nucleus Acc)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	6952,595	1	6952,595	521,6942	0,000000
STRESS	20,391	1	20,391	1,5300	0,223145
TREATMENT	0,175	1	0,175	0,0131	0,909320
STRESS*TREATMENT	17,167	1	17,167	1,2881	0,262982
Error	546,405	41	13,327		

Appendix H6: OXR-1 protein levels in the PFC

	OXR-1 protein levels in the PFC			
	Stress	Treatment	Stress x Treatment	Pixel Total (%)
1	NMS	nL	nMSnL	18,12
2	nMS	nL	nMSnL	8,32
3	nMS	nL	nMSnL	3,65
4	nMS	nL	nMSnL	11,36
5	nMS	nL	nMSnL	4,07
6	nMS	nL	nMSnL	8,42
7	nMS	nL	nMSnL	8,56
8	nMS	nL	nMSnL	8,07
9	nMS	nL	nMSnL	8,94111798
10	nMS	nL	nMSnL	25,9645639
11	nMS	nL	nMSnL	8,99554586
12	nMS	L	nMSL	16,14
13	nMS	L	nMSL	6,63
14	nMS	L	nMSL	11,18
15	nMS	L	nMSL	21,99
16	nMS	L	nMSL	12,53
17	nMS	L	nMSL	17,63
18	nMS	L	nMSL	16,46
19	nMS	L	nMSL	11,68
20	nMS	L	nMSL	13,4175287
21	MS	nL	MSnL	11,22
22	MS	nL	MSnL	19,13
23	MS	nL	MSnL	11,8
24	MS	nL	MSnL	17,26
25	MS	nL	MSnL	19,63
26	MS	nL	MSnL	16,8
27	MS	nL	MSnL	19,62

	OXR-1 protein levels in the PFC			
	Stress	Treatment	Stress x Treatment	Pixel Total (%)
28	MS	nL	MSnL	13,03
29	MS	nL	MSnL	11,7275855
30	MS	L	MSL	11
31	MS	L	MSL	9,45
32	MS	L	MSL	10,87
33	MS	L	MSL	11,9
34	MS	L	MSL	11,65
35	MS	L	MSL	9,26
36	MS	L	MSL	16,29
37	MS	L	MSL	6,28
38	MS	L	MSL	9,22682907



Breakdown Table of Descriptive Statistics (08 SEP 2014 OREXIN RECEPTORS STATS)
N=38 (No missing data in dep. var. list)

Stress	Treatment	Pixel Total (%) Means	Pixel Total (%) N	Pixel Total (%) Std.Dev.	Pixel Total (%) Std.Err.
NMS	nL	10,40648	11	6,399756	1,929599
NMS	L	14,18417	9	4,436987	1,478996
MS	nL	15,57973	9	3,610384	1,203461
MS	L	10,65854	9	2,716541	0,905514
All Groups		12,58614	38	4,985704	0,808788

Univariate Tests of Significance for Pixel Total (%) (08 SEP 2014 OREXIN RECEPTORS STATS)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	6089,863	1	6089,863	283,4902	0,000000
Stress	6,399	1	6,399	0,2979	0,588782
Treatment	3,082	1	3,082	0,1435	0,707202
Stress*Treatment	178,367	1	178,367	8,3032	0,006809
Error	730,379	34	21,482		

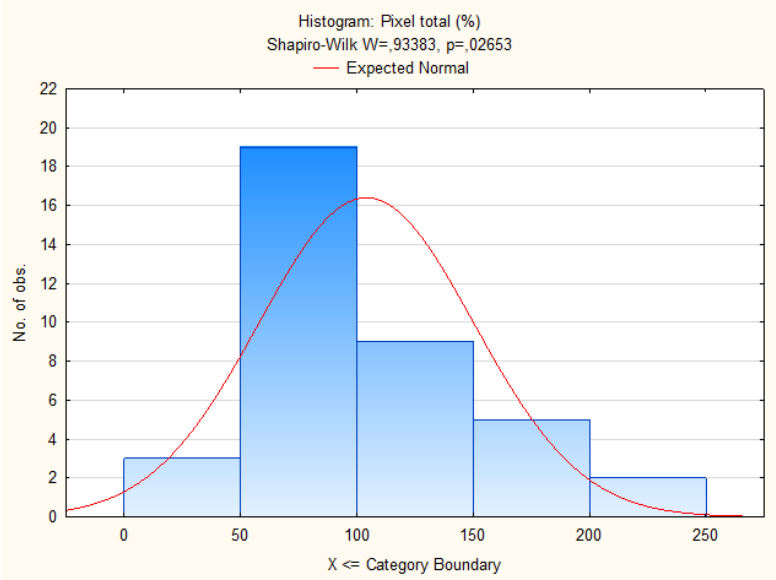
Duncan test; variable Pixel Total (%) (08 SEP 2014 OREXIN RECEPTORS STATS)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = 21,482, df = 34,000

Cell No.	Stress	Treatment	{1} 10,406	{2} 14,184	{3} 15,580	{4} 10,659
1	NMS	nL		0,102813	0,031570	0,906804
2	NMS	L	0,102813		0,517802	0,107954
3	MS	nL	0,031570	0,517802		0,034958
4	MS	L	0,906804	0,107954	0,034958	

OXR1 protein levels normalised against p38

	OXR1 protein levels normalised against p38			
	Stress	Treatment	Stress x Treatment	Pixel total (%)
1	NMS	nL	nMSnL	96,5370272
2	nMS	nL	nMSnL	70,2702703
3	nMS	nL	nMSnL	21,6745843
4	nMS	nL	nMSnL	104,220183
5	nMS	nL	nMSnL	23,7456243
6	nMS	nL	nMSnL	73,8596491
7	nMS	nL	nMSnL	83,3495618
8	nMS	nL	nMSnL	62,1247113
9	nMS	nL	nMSnL	56,0869108
10	nMS	nL	nMSnL	232,408046
11	nMS	nL	nMSnL	83,4032134
12	nMS	L	nMSL	125,407925
13	nMS	L	nMSL	72,6177437
14	nMS	L	nMSL	80,3738318
15	nMS	L	nMSL	217,29249
16	nMS	L	nMSL	97,8142077
17	nMS	L	nMSL	164,1527
18	nMS	L	nMSL	151,425943
19	nMS	L	nMSL	92,0409771
20	nMS	L	nMSL	92,4214056
21	MS	nL	MSnL	85,1289833
22	MS	nL	MSnL	171,569507
23	MS	nL	MSnL	100,084818
24	MS	nL	MSnL	139,193548
25	MS	nL	MSnL	156,539075
26	MS	nL	MSnL	146,853147
27	MS	nL	MSnL	161,216105

28	MS	nL	MSnL	88,1000676
29	MS	nL	MSnL	104,725976
30	MS	L	MSL	107,212476
31	MS	L	MSL	73,8858483
32	MS	L	MSL	96,1946903
33	MS	L	MSL	93,4799686
34	MS	L	MSL	100,344531
35	MS	L	MSL	75,1623377
36	MS	L	MSL	133,634126
37	MS	L	MSL	44,7931526
38	MS	L	MSL	77,2612586



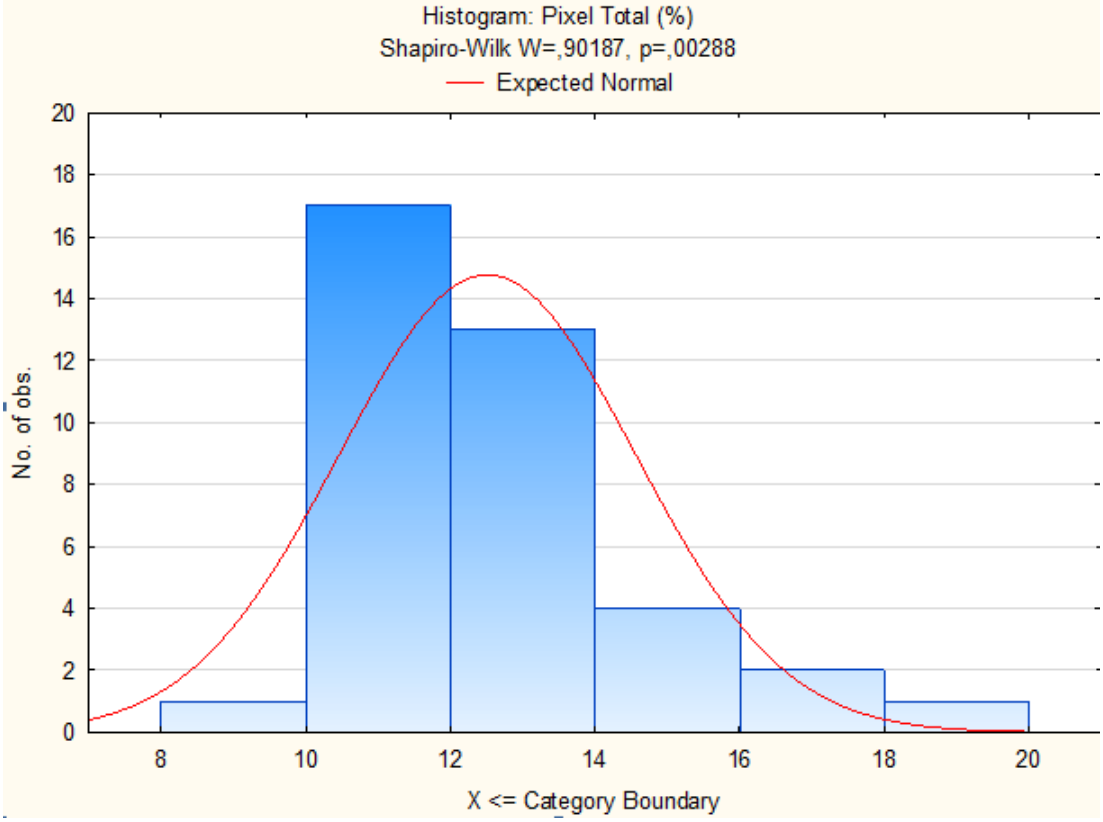
Multiple Comparisons p values (2-tailed); Pixel total (%) (08 SEP 2014 OREXIN RECEPTORS STATS)
Independent (grouping) variable: Stress x Treatment
Kruskal-Wallis test: H (3, N= 38) =10,08993 p =,0178

Depend.: Pixel total (%)	nMSnL R:12,455	nMSL R:23,111	MSnL R:27,111	MSL R:16,889
nMSnL		0,197318	0,020059	1,000000
nMSL	0,197318		1,000000	1,000000
MSnL	0,020059	1,000000		0,306147
MSL	1,000000	1,000000	0,306147	

P38 protein levels in the PFC

	P38 protein levels in the PFC			
	Stress	Treatment	Stress x Treatment	Pixel Total (%)
1	nMS	nL	nMSnL	18,77
2	nMS	nL	nMSnL	11,84
3	nMS	nL	nMSnL	16,84
4	nMS	nL	nMSnL	10,9
5	nMS	nL	nMSnL	17,14
6	nMS	nL	nMSnL	11,4
7	nMS	nL	nMSnL	10,27
8	nMS	nL	nMSnL	12,99
9	nMS	nL	nMSnL	15,9415412
10	nMS	nL	nMSnL	11,1719729
11	nMS	nL	nMSnL	10,7856106
12	nMS	L	nMSL	12,87
13	nMS	L	nMSL	9,13
14	nMS	L	nMSL	13,91
15	nMS	L	nMSL	10,12
16	nMS	L	nMSL	12,81
17	nMS	L	nMSL	10,74
18	nMS	L	nMSL	10,87
19	nMS	L	nMSL	12,69
20	nMS	L	nMSL	14,5177717
21	MS	nL	MSnL	13,18
22	MS	nL	MSnL	11,15
23	MS	nL	MSnL	11,79
24	MS	nL	MSnL	12,4
25	MS	nL	MSnL	12,54
26	MS	nL	MSnL	11,44

	P38 protein levels in the PFC			
	Stress	Treatment	Stress x Treatment	Pixel Total (%)
27	MS	nL	MSnL	12,17
28	MS	nL	MSnL	14,79
29	MS	nL	MSnL	11,198354
30	MS	L	MSL	10,26
31	MS	L	MSL	12,79
32	MS	L	MSL	11,3
33	MS	L	MSL	12,73
34	MS	L	MSL	11,61
35	MS	L	MSL	12,32
36	MS	L	MSL	12,19
37	MS	L	MSL	14,02
38	MS	L	MSL	11,9423748



Breakdown Table of Descriptive Statistics (08 SEP 2014 OREXIN RECEPTORS STATS)
N=38 (No missing data in dep. var. list)

Stress	Treatment	Pixel Total (%) Means	Pixel Total (%) N	Pixel Total (%) Std.Dev.
nMS	nL	13,45901	11	3,090744
nMS	L	11,96197	9	1,819999
MS	nL	12,29537	9	1,150795
MS	L	12,12915	9	1,054680
All Groups		12,51388	38	2,052347

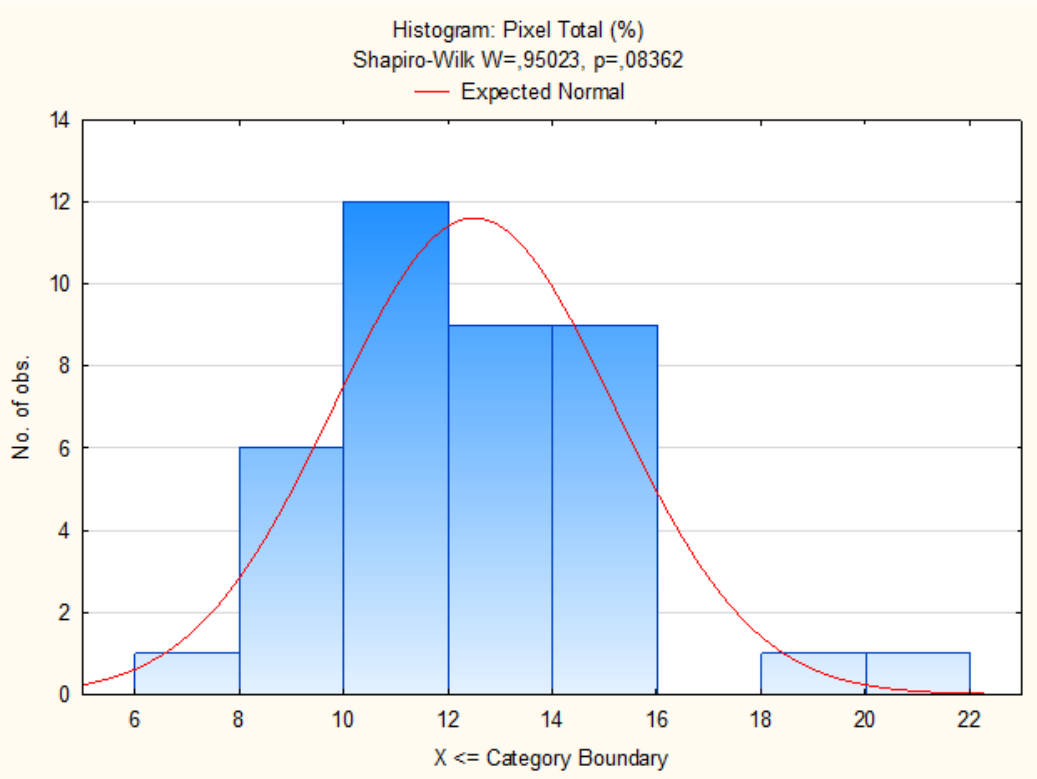
Multiple Comparisons z' values; Pixel Total (%) (08 SEP 2014 OREXIN RECEPTORS STATS)
Independent (grouping) variable: Stress x Treatment
Kruskal-Wallis test: H (3, N= 38) =,5441050 p =,9091

Depend.: Pixel Total (%)	nMSnL R:21,182	nMSL R:17,556	MSnL R:19,778	MSL R:19,111
nMSnL		0,725986	0,281092	0,414560
nMSL	0,725986		0,424190	0,296933
MSnL	0,281092	0,424190		0,127257
MSL	0,414560	0,296933	0,127257	

Appendix H7: OXR-2 protein levels in the PFC

	OXR-2 protein levels in the PFC			
	Stress	Treatment	Stress x Treatment	Pixel Total (%)
1	nMS	nL	nMSnL	20,72
2	nMS	nL	nMSnL	9,93
3	nMS	nL	nMSnL	12,86
4	nMS	nL	nMSnL	9,97
5	nMS	nL	nMSnL	7,47
6	nMS	nL	nMSnL	11,54
7	nMS	nL	nMSnL	11,6
8	nMS	nL	nMSnL	8,52
9	nMS	nL	nMSnL	12,8385741
10	nMS	nL	nMSnL	11,9178652
11	nMS	nL	nMSnL	9,85887009
12	nMS	L	nMSL	10,54
13	nMS	L	nMSL	10,99
14	nMS	L	nMSL	11,53
15	nMS	L	nMSL	15
16	nMS	L	nMSL	14,2
17	nMS	L	nMSL	14,9
18	nMS	L	nMSL	12,53
19	nMS	L	nMSL	12,79
20	nMS	L	nMSL	11,4413762
21	MS	nL	MSnL	10,61
22	MS	nL	MSnL	15,32
23	MS	nL	MSnL	11,57
24	MS	nL	MSnL	15,42
25	MS	nL	MSnL	15,14
26	MS	nL	MSnL	14,58
27	MS	nL	MSnL	13,35

	OXR-2 protein levels in the PFC			
	Stress	Treatment	Stress x Treatment	Pixel Total (%)
28	MS	nL	MSnL	14,9
29	MS	nL	MSnL	18,9662368
30	MS	nL	MSnL	10,0481724
31	MS	L	MSL	9,2
32	MS	L	MSL	12,69
33	MS	L	MSL	10,38
34	MS	L	MSL	13,27
35	MS	L	MSL	9,1
36	MS	L	MSL	13,06
37	MS	L	MSL	11,78
38	MS	L	MSL	14,52
39	MS	L	MSL	12,4289052



Breakdown Table of Descriptive Statistics (08 SEP 2014 OREXIN RECEPTORS STATS)
N=39 (No missing data in dep. var. list)

Stress	Treatment	Pixel Total (%) Means	Pixel Total (%) N	Pixel Total (%) Std.Dev.	Pixel Total (%) Std.Err.
nMS	nL	11,56594	11	3,484373	1,050578
nMS	L	12,65793	9	1,693510	0,564503
MS	nL	13,99044	10	2,672989	0,845273
MS	L	11,82543	9	1,883995	0,627998
All Groups		12,49949	39	2,681235	0,429341

Univariate Tests of Significance for Pixel Total (%) (08 SEP 2014 OREXIN RECEPTORS STATS)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	6060,968	1	6060,968	894,8845	0,000000
Stress	6,135	1	6,135	0,9058	0,347761
Treatment	2,787	1	2,787	0,4115	0,525399
Stress*Treatment	25,677	1	25,677	3,7912	0,059583
Error	237,052	35	6,773		

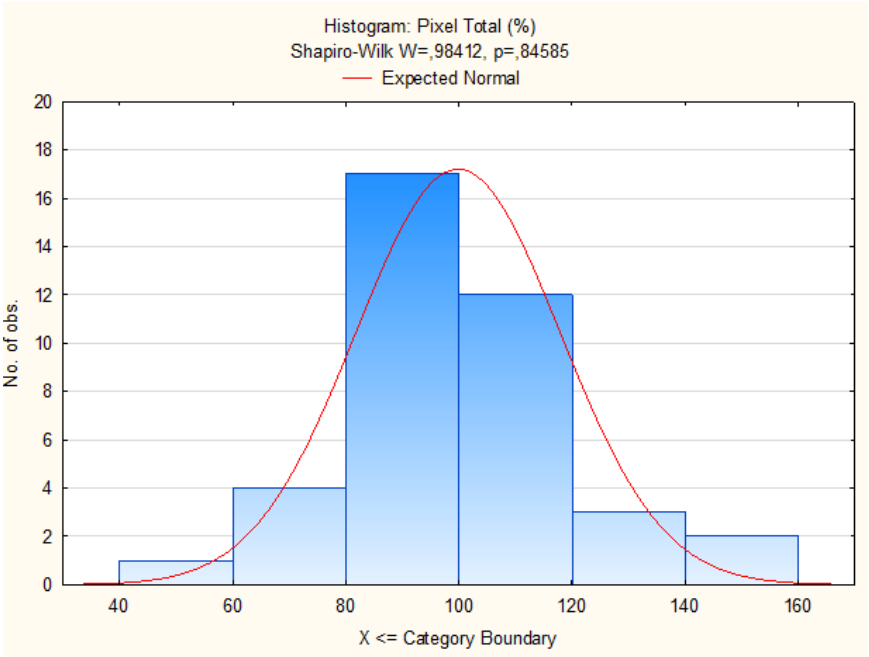
Duncan test; variable Pixel Total (%) (08 SEP 2014 OREXIN RECEPTORS STATS)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = 6,7729, df = 35,000

Cell No.	Stress	Treatment	{1} 11,566	{2} 12,658	{3} 13,990	{4} 11,825
1	nMS	nL		0,391351	0,067761	0,827739
2	nMS	L	0,391351		0,267720	0,486337
3	MS	nL	0,067761	0,267720		0,091543
4	MS	L	0,827739	0,486337	0,091543	

OXR-2 protein levels in the PFC normalised against p38

	OXR-2 protein levels in the PFC normalised against p38			
	Stress	Treatment	Stress x Treatment	Pixel Total (%)
1	nMS	nL	nMSnL	144,289694
2	nMS	nL	nMSnL	85,0171233
3	nMS	nL	nMSnL	98,393267
4	nMS	nL	nMSnL	83,6409396
5	nMS	nL	nMSnL	57,5944487
6	nMS	nL	nMSnL	96,1666667
7	nMS	nL	nMSnL	77,1789754
8	nMS	nL	nMSnL	73,5751295
9	nMS	nL	nMSnL	88,440776
10	nMS	nL	nMSnL	98,1561652
11	nMS	nL	nMSnL	81,1885423
12	nMS	L	nMSL	105,717151
13	nMS	L	nMSL	87,0839937
14	nMS	L	nMSL	97,2175379
15	nMS	L	nMSL	119,142176
16	nMS	L	nMSL	113,964687
17	nMS	L	nMSL	121,632653
18	nMS	L	nMSL	100,64257
19	nMS	L	nMSL	104,153094
20	nMS	L	nMSL	97,9182954
21	MS	nL	MSnL	92,4216028
22	MS	nL	MSnL	111,743253
23	MS	nL	MSnL	92,2647528
24	MS	nL	MSnL	125,264013
25	MS	nL	MSnL	106,770099
26	MS	nL	MSnL	118,729642
27	MS	nL	MSnL	104,870385
28	MS	nL	MSnL	121,831562

29	MS	nL	MSnL	140,849938
30	MS	nL	MSnL	96,7631064
31	MS	L	MSL	76,2220381
32	MS	L	MSL	89,9362155
33	MS	L	MSL	91,4537445
34	MS	L	MSL	92,4094708
35	MS	L	MSL	76,3422819
36	MS	L	MSL	109,380235
37	MS	L	MSL	108,371665
38	MS	L	MSL	113,26053
39	MS	L	MSL	94,4156921



Breakdown Table of Descriptive Statistics (08 SEP 2014 OREXIN RECEPTORS STATS)
N=39 (No missing data in dep. var. list)

Stress	Treatment	Pixel Total (%) Means	Pixel Total (%) N	Pixel Total (%) Std.Dev.	Pixel Total (%) Std.Err.
nMS	nL	89,4220	11	21,79061	6,570117
nMS	L	105,2747	9	11,22638	3,742125
MS	nL	111,1508	10	15,72753	4,973480
MS	L	94,6435	9	13,52723	4,509078
All Groups		99,8568	39	18,08512	2,895937

Univariate Tests of Significance for Pixel Total (%) (08 SEP 2014 OREXIN RECEPTORS STATS)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	388237,5	1	388237,5	1438,428	0,000000
Stress	298,1	1	298,1	1,105	0,300481
Treatment	1,0	1	1,0	0,004	0,950924
Stress*Treatment	2534,7	1	2534,7	9,391	0,004181
Error	9446,6	35	269,9		

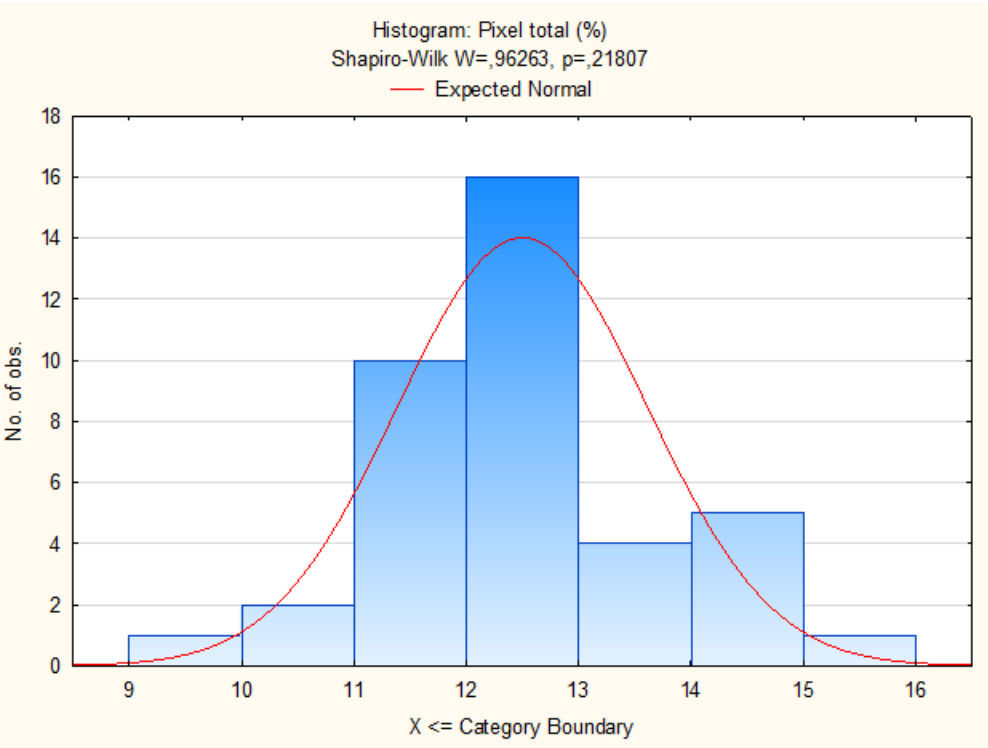
Duncan test; variable Pixel Total (%) (08 SEP 2014 OREXIN RECEPTORS STATS)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = 269,90, df = 35,000

Cell No.	Stress	Treatment	{1} 89,422	{2} 105,27	{3} 111,15	{4} 94,644
1	nMS	nL		0,051199	0,010243	0,489122
2	nMS	L	0,051199		0,436738	0,163478
3	MS	nL	0,010243	0,436738		0,042597
4	MS	L	0,489122	0,163478	0,042597	

P38 Protein levels in the PFC (OXR-2)

	P38 Protein levels in the PFC (OXR-2)			
	Stress	Treatment	Stress x Treatment	Var4
1	nMS	nL	nMSnL	14,36
2	nMS	nL	nMSnL	11,68
3	nMS	nL	nMSnL	13,07
4	nMS	nL	nMSnL	11,92
5	nMS	nL	nMSnL	12,97
6	nMS	nL	nMSnL	12
7	nMS	nL	nMSnL	15,03
8	nMS	nL	nMSnL	11,58
9	nMS	nL	nMSnL	14,5165779
10	nMS	nL	nMSnL	12,1417388
11	nMS	nL	nMSnL	12,1431791
12	nMS	L	nMSL	9,97
13	nMS	L	nMSL	12,62
14	nMS	L	nMSL	11,86
15	nMS	L	nMSL	12,59
16	nMS	L	nMSL	12,46
17	nMS	L	nMSL	12,25
18	nMS	L	nMSL	12,45
19	nMS	L	nMSL	12,28
20	nMS	L	nMSL	11,6846154
21	MS	nL	MSnL	11,48
22	MS	nL	MSnL	13,71
23	MS	nL	MSnL	12,54
24	MS	nL	MSnL	12,31
25	MS	nL	MSnL	14,18
26	MS	nL	MSnL	12,28

	P38 Protein levels in the PFC (OXR-2)			
	Stress	Treatment	Stress x Treatment	Var4
27	MS	nL	MSnL	12,73
28	MS	nL	MSnL	12,23
29	MS	nL	MSnL	13,4655628
30	MS	nL	MSnL	10,3843011
31	MS	L	MSL	12,07
32	MS	L	MSL	14,11
33	MS	L	MSL	11,35
34	MS	L	MSL	14,36
35	MS	L	MSL	11,92
36	MS	L	MSL	11,94
37	MS	L	MSL	10,87
38	MS	L	MSL	12,82
39	MS	L	MSL	13,1640249



Breakdown Table of Descriptive Statistics (08 SEP 2014 OREXIN RECEPTORS STATS)
N=39 (No missing data in dep. var. list)

Stress	Treatment	Pixel total (%) Means	Pixel total (%) N	Pixel total (%) Std.Dev.	Pixel total (%) Std.Err.
nMS	nL	12,85559	11	1,242693	0,374686
nMS	L	12,01829	9	0,830998	0,276999
MS	nL	12,53099	10	1,102406	0,348611
MS	L	12,51156	9	1,194159	0,398053
All Groups		12,49974	39	1,109804	0,177711

Univariate Tests of Significance for Pixel total (%) (08 SEP 2014 OREXIN RECEPTORS STATS)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	6031,132	1	6031,132	4873,570	0,000000
Stress	0,069	1	0,069	0,056	0,814896
Treatment	1,777	1	1,777	1,436	0,238897
Stress*Treatment	1,619	1	1,619	1,308	0,260453
Error	43,313	35	1,238		

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